UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS
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TESIS DOCTORAL

Propiedades inmunomoduladoras de un surfactante pulmonar sintético basado en la proteína recombinante humana SP-C: mecanismo de acción y papel de sus componentes.

Inmunomodulatory properties of a synthetic pulmonary surfactant based on human recombinant protein SP-C : mechanism of action and role of its components

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

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DOCTORAL THESIS OF

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A mi familia

A Alex

The research for this doctoral thesis has been performed at the Department of Biochemistry and Molecular Biology I of the Complutense University of Madrid, under the supervision of Professor Cristina Casals Carro.

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ΔH	Enthalpy of the gel-to-liquid phase transition
$\Delta T_{1/2}$	Temperature width at half-height of the DSC peak
γ	Surface tension
ABCA3	ATP-binding cassette transporter A3
AEC	Alveolar epithelial cell
ALI	Acute lung injury
AM	Alveolar macrophage
AP1	Activating protein 1
ARDS	Acute respiratory distress syndrome
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CD	Cluster of differentiation
CD200R	Cluster of differentiation 200 receptor
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT-enhancer box binding protein
COX2	Cyclooxygenase 2
C_p	Excess heat capacity
CRD	Carbohydrate recognition domain
CREB	cAMP-response element binding protein
CXCL10, 11	C-X-C motif chemokines 10 and 11
DAMPs	Damage-associated molecular patterns
DAPI	4’,6-diamidino-2-phenylindole
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

DLS	Dynamic Light Scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphate
DPPC	Dipalmitoylphosphatidylcholine
DSC	Differential scanning calorimetry
dsRNA	Double-stranded ribonucleic acid
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
ERK	Extracellular signal-regulated kinase
FAM	Carboxyfluorescein
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulation factor
GSK3	Glycogen synthase kinase 3
GUV	Giant Unilamellar Vesicle
HRP	Horseradish peroxidase
IFIT	Interferon-induced protein with tetratricopeptide repeats
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor

ISG15	Interferon-stimulated gene 15
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
ITO	Indium tin oxide
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
L_α	Liquid-crystalline phase
L_β	Gel phase
LAL	Limulus Amebocyte Lysate
LB	Lamellar body
LBP	Lipopolysaccharide-binding protein
L_o	Liquid-ordered phase
LPS	Lipopolysaccharide
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MGB	Minor groove binder
MLV	Multilamellar vesicle
moi	Multiplicity of infection
mTORC2	Mammalian target of rapamycin complex 2
MVB	Multivesicular body
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
NIK	Nuclear factor-κB-inducing kinase
NMR	Nuclear magnetic resonance
$P_{\beta'}$	Ripple phase
PA	Palmitic acid

PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDK1	Phosphoinositide-dependent kinase 1
PE	Phosphatidylethanolamine
pfu	Plaque-forming units
PG	Phosphatidylglycerol
PGE2	Prostaglandin E2
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PLs	Phospholipids
PMSF	Phenylmethylsulfonyl fluoride
POPC	Palmitoyloleoylphosphatidylcholine
POPG	Palmitoyloleoylphosphatidylglycerol
PPARγ	Peroxisome proliferator-activated receptor gamma
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real-time polymerase chain reaction
RANTES	Regulated on activation normal T cell expressed and secreted
RDS	Respiratory distress syndrome
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus

SD	Standard deviation
SDS	Sodium dodecyl-sulphate
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SE	Standard error
SIRPα	Signal inhibitory regulatory protein alpha
SOCS	Suppressor of cytokine signaling
SP-A, -B, -C, -D	Surfactant protein A, B, C, D
sPL	Synthetic surfactant without human recombinant SP-C
sSPC	Synthetic surfactant containing human recombinant SP-C
STAT	Signal transducer and activator of transcription
SUV	Small Unilamellar Vesicle
TBS	Tris buffered saline
TCF	T-cell factor
TGF-β	Transforming growth factor beta
T_H	Helper T cell
TLR	Toll-like receptor
T_m	Phase transition temperature
TM	Tubular myelin
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TNFAIP3	Tumor necrosis factor alpha-induced protein 3
UPL	Universal Probe Library
Ym1	Chitinase 3-like-3

List of abbreviations

RESUMEN

Resumen

INTRODUCCIÓN

Los pulmones se encuentran continuamente expuestos a gran cantidad de alérgenos, patógenos y partículas que escapan a los mecanismos de defensa de las vías respiratorias superiores. Para permanecer sanos, los pulmones deben tener una respuesta inmune capaz de matar y eliminar a los agentes patógenos sin desencadenar una reacción inflamatoria exacerbada que podría dañar el tejido alveolar y dificultar el intercambio gaseoso.

En el espacio alveolar, los macrófagos alveolares (AMs) y las células epiteliales alveolares (AECs) se encuentran en contacto continuo con el surfactante pulmonar, una compleja red extracelular de membranas sintetizada y secretada por las AECs de tipo II. El surfactante está formado por 90% de lípidos (principalmente fosfolípidos) y contiene cuatro proteínas asociadas [proteínas del surfactante A, B, C y D (SP-A, SP-B, SP-C y SP-D)] (Casals and Cañadas 2012). La función principal del surfactante pulmonar es reducir la tensión superficial en la interfase aire-líquido para evitar el colapso alveolar al final de la espiración. La deficiencia de surfactante en pulmones inmaduros es la causa principal del síndrome de distrés respiratorio (RDS) neonatal (Whitsett and Weaver 2002).

Además de su relevante función biofísica, el surfactante pulmonar desarrolla un papel esencial en la defensa inmune del pulmón (Wright 2005; Chroneos et al. 2010; Ariki et al. 2012; Glasser and Mallampalli 2012). Aunque se está estudiando el mecanismo de la acción inmunomoduladora de los distintos componentes del surfactante, todavía hay varios puntos sin resolver. Además, en el espacio alveolar, los AMs y las AECs tipo II endocitan parte del surfactante pulmonar para asegurar su reciclaje y su degradación (Casals and Cañadas 2012; Agassandian and Mallampalli 2013). El efecto de los componentes del surfactante internalizados sobre el fenotipo o la respuesta inmune de los AMs o las AECs aún no se conoce.

Actualmente, se están desarrollando surfactantes pulmonares sintéticos para tratar el RDS. Entre ellos, el surfactante pulmonar sintético basado en la proteína recombinante humana SP-C (sSPC), que está compuesto de 98% de lípidos [dipalmitoilfosfatidilcolina (DPPC)/palmitoiloleilfosfatidilglicerol (POPG)/ácido palmítico (PA) en proporción 2.3:1:0.16 en peso] y 2% de SP-C recombinante humana. El surfactante sSPC ha sido efectivo en el tratamiento de modelos animales de daño pulmonar (Lewis et al. 1999; Spragg et al. 2000; Ikegami and Jobe 2002) y de pacientes con síndrome de distrés

respiratorio agudo (ARDS) (Spragg et al. 2003; Spragg et al. 2004; Markart et al. 2007). Además, Spragg y colaboradores (2003) observaron que el surfactante sintético basado en SP-C exhibía una potencial acción anti-inflamatoria ya que el tratamiento con dicho surfactante redujo los niveles de IL-6 en lavados broncoalveolares de pacientes con ARDS. Además, el surfactante sintético sSPC redujo la expresión de TNF-α en una línea celular de monocitos humanos estimulados con lipopolisacárido bacteriano (LPS) (Wemhöner et al. 2009). Un mayor conocimiento del potencial papel modulador de los distintos componentes del surfactante pulmonar sintético basado en SP-C sobre la respuesta de células alveolares a endotoxinas o virus es importante para entender sus posibles efectos beneficiosos en el tratamiento de enfermedades.

OBJETIVOS

El **objetivo principal** de esta tesis fue estudiar las propiedades inmunoreguladoras del surfactante pulmonar sintético basado en la proteína recombinante humana SP-C y explicar el mecanismo de acción de cada uno de sus componentes. Para llevar a cabo esta tarea, se utilizaron dos estímulos que inducen una fuerte respuesta pro-inflamatoria en AMs y AECs:

- LPS bacteriano: un componente de la membrana externa de las bacterias Gram-negativas que promueve el desarrollo de daño pulmonar agudo o ARDS como consecuencia de infecciones pulmonares bacterianas o sepsis (Matute-Bello et al. 2008).
- Virus respiratorio sincitial (RSV): un virus respiratorio común y altamente contagioso que infecta las AECs y las células residentes del sistema inmune, induciendo una fuerte respuesta pro-inflamatoria que ayuda a eliminar el virus pero que también puede ser perjudicial para el huésped (Lotz and Peebles 2012).

Esta **tesis está compuesta por tres capítulos, cuyos objetivos concretos son**:

1) Determinar el efecto anti-inflamatorio extracelular del surfactante sintético basado en SP-C en la respuesta inflamatoria de macrófagos alveolares y peritoneales de ratón estimulados con LPS (capítulo 1).

2) Evaluar la acción inmunomoduladora intracelular de vesículas del surfactante sintético basado en SP-C internalizadas por macrófagos alveolares sobre el estado de activación dichas células tras ser estimuladas con LPS (capítulo 2).

3) Investigar la función inmunoreguladora intracelular de vesículas del surfactante sintético internalizadas sobre la respuesta inmune de células epiteliales alveolares humanas (A549) infectadas con el virus respiratorio sincitial humano (capítulo 3).

RESULTADOS Y CONCLUSIONES

❖ Capítulo 1

Para evaluar el efecto anti-inflamatorio extracelular del surfactante sintético sobre la respuesta inflamatoria de macrófagos alveolares y peritoneales estimulados con LPS, se han utilizado dos líneas celulares de macrófagos murinos: macrófagos alveolares MH-S, y macrófagos peritoneales RAW 264.7. Las vesículas de surfactante sintético con (sSPC) o sin (sPL) la proteína recombinante humana SP-C se añadieron a las células simultáneamente con el LPS.

El surfactante sintético inhibió la respuesta pro-inflamatoria de AMs estimulados con LPS, ya que bloqueó la activación de las tres principales rutas de señalización comúnmente activadas por LPS (la cascada de proteína quinasas activadas por mitógenos (MAPKs), la vía de la fosfatidilinositol-3-quinasa (PI3K)/Akt, y la activación del factor nuclear-κB (NF-κB)). Consecuentemente, el surfactante sintético disminuyó la producción de los mediadores pro-inflamatorios TNF-α y óxido nítrico sintasa inducible (iNOS) por AMs estimulados con LPS. Esta acción inmunomoduladora no se produjo de forma selectiva en macrófagos alveolares, ya que el surfactante sintético también atenuó la secreción de TNF-α en macrófagos peritoneales. La acción anti-inflamatoria del surfactante sintético disminuyó conforme las vesículas de surfactante se endocitaron por los AMs a través de un mecanismo dependiente de clatrina. Dado que los resultados obtenidos indicaron que el surfactante sintético no secuestra moléculas de LPS, los resultados sugieren que actúa directamente en la superficie de los AMs, probablemente impidiendo la unión del LPS a su receptor.

Con respecto al papel de los componentes lipídico y proteico del surfactante sintético en su acción inmunomoduladora, la proteína SP-C solamente está implicada en la reducción de la expresión de mRNA y de los niveles proteicos de iNOS, siendo el componente lipídico del surfactante el mayor responsable de la inhibición de la respuesta pro-inflamatoria inducida por LPS. Entre los distintos componentes lipídicos, demostramos que POPG es esencial para la acción anti-inflamatoria extracelular del surfactante, ya que vesículas lipídicas que carecían de POPG no eran capaces de disminuir la secreción de TNF-α tras la estimulación de las células con LPS. Además, demostramos que las vesículas de DPPC/POPG/PA ejercían una inhibición significativamente mayor que aquellas de DPPC/POPG sobre la producción de TNF-α por macrófagos alveolares estimulados con LPS. Demostramos que la presencia de PA en vesículas de DPPC/POPG/PA aumenta la segregación de dominios ordenados y desordenados en la membrana del surfactante, con el consiguiente enriquecimiento de POPG en los dominios fluidos desordenados, lo que incrementa su acción anti-inflamatoria.

Conclusiones: Los resultados obtenidos indicaron que el componente lipídico del surfactante, y en concreto el POPG, es el responsable de la inhibición de la respuesta pro-inflamatoria de macrófagos alveolares estimulados con LPS. Este efecto es extracelular ya que desaparece cuando las vesículas son endocitadas por las células. Dado que las vesículas de surfactante no secuestran moléculas de LPS, su acción anti-inflamatoria parece llevarse a cabo directamente en la superficie de los macrófagos, probablemente impidiendo la unión del LPS a su receptor, lo que explica el bloqueo que ejerce el surfactante sobre todas la vías de señalización activadas por LPS. Por otro lado, la proteína recombinante humana SP-C disminuyó únicamente la producción de iNOS por macrófagos alveolares estimulados con LPS. Nuestros resultados también demuestran por primera vez que la presencia del componente lipídico PA en la membrana del surfactante sintético es esencial para aumentar la segregación de dominios ordenados/desordenados en la membrana del surfactante, lo que determina el enriquecimiento en POPG en dominios desordenados y la mejora de sus propiedades inmunoreguladoras.

❖ Capítulo 2

Para analizar la potencial acción inmunomoduladora intracelular de vesículas de surfactante sintético internalizadas por macrófagos alveolares, las células se preincubaron con vesículas de sSPC o sPL durante 18 o 24 horas antes de la estimulación con LPS.

En primer lugar, los estudios de microscopía confocal y citometría de flujo revelaron que las vesículas unilamelares pequeñas de surfactante sintético se endocitan eficazmente por las células tras una preincubación de 18-24 horas. En contraste, las vesículas multilamelares de sSPC y sPL sólo se internalizan parcialmente en el mismo periodo, por lo que para llevar a cabo este estudio se utilizaron exclusivamente vesículas unilamelares de surfactante sintético.

Los resultados indicaron que, por un lado, los lípidos del surfactante sintético internalizados disminuyeron la expresión y liberación de TNF-α y la secreción de la citoquina anti-inflamatoria IL-10 por macrófagos alveolares estimulados con LPS, siendo este efecto muy pronunciado en el caso de la secreción de TNF-α (78 ± 3% de inhibición). Por otro lado, las vesículas lipídicas endocitadas incrementaron la expresión inducida por LPS de varios marcadores pro-inflamatorios [factor regulador de interferón (IRF)1, quimoquinas con motivos C-X-C 10 y 11 (CXCL10 y CXCL11) y CD80], y de mediadores que pueden tener actividad pro- o anti-inflamatoria según el contexto [la ciclooxigenasa 2 (COX2) y el supresor de la señalización por citoquinas 3 (SOCS 3)].

Con respecto a las rutas de señalización que podrían estar implicadas, demostramos que las vesículas lipídicas internalizadas inhibieron la activación de NF-κB y la fosforilación y activación de Akt en macrófagos estimulados con LPS. Además promovieron la activación de la glucógeno sintasa quinasa 3 (GSK3), probablemente como consecuencia de la inhibición de Akt, proteína quinasa implicada en la fosforilación e inactivación de GSK3. Sin embargo, las vesículas lipídicas internalizadas no afectan a otras cascadas de señalización activadas por LPS, tal como p38 MAPK y la quinasa regulada por señales extracelulares (ERK), indicando que estás vías de señalización no están implicadas en la acción anti-inflamatoria del surfactante sintético internalizado.

Los experimentos de inhibición de GSK3 con cloruro de litio demostraron que la acción inmunoreguladora ejercida por los lípidos internalizados está mediada, al menos en parte, por la activación de GSK3, probablemente como consecuencia de la inhibición de la

ruta de PI3K/Akt. Aunque el efecto de los lípidos internalizados sobre la disminución de la producción de TNF-α podría también deberse a la inhibición que éstos ejercen en la ruta de NF-κB.

En cuanto al papel inmunomodulador del componente proteico del complejo, los resultados indican que la proteína SP-C internalizada por los macrófagos alveolares aumenta tanto la expresión de moléculas implicadas en la activación alternativa de los macrófagos [IL-4 y el receptor CD200R3] como la producción del marcador pro-inflamatorio iNOS.

Conclusiones: Una vez que el surfactante sintético es endocitado por los macrófagos alveolares, tanto la proteína recombinante humana SP-C como el componente lipídico de dicho complejo ejercen acciones inmunoreguladoras en el interior de las células, a través de la expresión de marcadores pro- y anti-inflamatorios, que modulan la respuesta celular a LPS. El balance final es la limitación de la respuesta pro-inflamatoria inducida por la endotoxina. Los resultados también demostraron por primera vez que el efecto intracelular del componente lipídico del surfactante está mediado, al menos en parte, por la activación de la proteína GSK3.

❖ Capítulo 3

Para examinar la función inmunoreguladora intracelular del surfactante sintético sobre la respuesta inmune de células epiteliales alveolares humanas (A549) infectadas con el virus respiratorio sincitial humano, las células se preincubaron con vesículas de sSPC o sPL durante 18 horas antes de ser infectadas o no con el virus.

El análisis de los niveles proteicos de SP-C en células A549 previamente preincubadas con surfactante sintético durante 18 horas confirmó la internación de dicho complejo por las células epiteliales humanas. Las vesículas de surfactante sintético internalizadas no tuvieron ningún efecto en la replicación viral en pneumocitos infectados con el RSV. Sin embargo, disminuyeron la expresión inducida por el RSV de moléculas antivirales [el gen estimulado por interferón 15 (ISG15) y la proteína inducida por interferón con repeticiones del tetratricopéptido (IFIT) 1], de la proteína inhibidora de los factores de transcripción NF-κB e IRF3 [la proteína 3 inducida por el factor de necrosis tumoral α (TNFAIP3)], y de

receptores celulares que reconocen el RNA viral de doble hebra [el receptor tipo Toll (TLR)3 y el gen inducible por ácido retinoico I (RIG-I)]. El surfactante sintético también inhibió la activación inducida por el RSV de ERK y de la ruta de PI3K/Akt. No obstante, las vesículas de surfactante sintético internalizadas no influyeron en la activación de la MAPK p38 ni de la ruta de NF-κB. Además, demostramos que es el componente lipídico del surfactante, y no el proteico, el responsable de la inhibición de la expresión de ISG15 y RIG-I, así como la inhibición de la activación de ERK en las células infectadas con el RSV.

Conclusiones: Los resultados indicaron que las vesículas de surfactante sintético internalizadas ejercen una acción anti-inflamatoria en el interior de células epiteliales alveolares humanas (A549) infectadas con el RSV, inhibiendo la expresión de mediadores inflamatorios y la activación de ERK y de la ruta de PI3K/Akt inducidas por el virus. Además, demostramos que el componente lipídico del surfactante sintético es el que lleva a cabo la inhibición de la expresión de ISG15 y RIG-I y de la activación de ERK en las células infectadas con el RSV.

SUMMARY

INTRODUCTION

The lungs are continuously exposed to a diverse array of inhaled particles, allergens and pathogens that escape the defense mechanisms of the upper respiratory airways. To remain healthy, the lungs must have an immune response able to kill and clear pathogens without triggering an excessive inflammatory response, which would damage alveolar tissues, compromising lung homeostasis and gas exchange.

In the alveolar space, alveolar macrophages (AMs) and alveolar epithelial cells (AECs) are continuously in contact with pulmonary surfactant, a complex network of extracellular membranes synthesized and secreted into the alveolar space by type II AECs. Surfactant consists of approximately 90% of lipids (mainly phospholipids) and contains four associated proteins [surfactant proteins A, B, C and D (SP-A, SP-B, SP-C and SP-D)] (Casals and Cañadas 2012). The main function of surfactant is to reduce the surface tension at the air-liquid interface in order to prevent the lungs from collapsing at the end of expiration. Surfactant deficiency in immature lungs is the main cause of neonatal respiratory distress syndrome (RDS) (Whitsett and Weaver 2002).

In addition to its biophysical relevance, surfactant has also been recognized to play an essential role in the immune host defense of the lungs (Wright 2005; Chroneos et al. 2010; Ariki et al. 2012; Glasser and Mallampalli 2012). Although the mechanism underlying the immunoregulatory action of surfactant components is being elucidated, some points are still unknown. Furthermore, in the alveolar space surfactant is endocytosed by AMs and type II pneumocytes in order to assure its degradation and recycling (Casals and Cañadas 2012; Agassandian and Mallampalli 2013). The effect of these internalized surfactant components on the phenotype or the immune response of AMs and AECs remains largely unknown.

Synthetic surfactants are currently being developed to treat RDS. Among them, a synthetic pulmonary surfactant based on recombinant human SP-C (sSPC) is composed of 98% of lipids by weight [dipalmitoylphosphatidylcholine (DPPC)/palmitoyloleoylphosphatidylglycerol (POPG)/palmitic acid (PA) 2.3:1:0.16 w/w] and 2% of recombinant human SP-C. sSPC surfactant has been shown to be effective in animal models of lung injury (Lewis et al. 1999; Spragg et al. 2000; Ikegami and Jobe 2002) and treating acute respiratory distress syndrome (ARDS) patients (Spragg et al. 2003; Spragg et al. 2004; Markart et al. 2007). Furthermore, Spragg et al. (2003) observed that the

synthetic surfactant based on recombinant human SP-C presented a potential anti-inflammatory action, since the treatment with sSPC decreased the levels of IL-6 in bronchoalveolar lavages of patients suffering ARDS (Spragg et al. 2003). In addition to that, sSPC synthetic surfactant was also shown to reduce TNF-α expression in a human monocytic cell line stimulated with bacterial lipopolysaccharide (LPS) (Wemhöner et al. 2009). A greater knowledge about the potential modulatory action of the different components present in the synthetic surfactant based on recombinant human SP-C on the immune response of alveolar cells to viruses or endotoxins is important in order to understand their possible beneficial effect on the treatment of respiratory diseases.

OBJECTIVES

The **main objective** of this thesis was to study the immunoregulatory properties of the synthetic surfactant based on recombinant human SP-C and to unravel new details about the mechanism of action of each of its components. In order to accomplish this task, we used two stimuli that have been reported to induce a strong pro-inflammatory response in AMs and AECs:

- Bacterial LPS, which is a component of the outer membrane of Gram-negative bacteria that can promote the development of acute lung injury and ARDS as a consequence of pulmonary bacterial infections or sepsis (Matute-Bello et al. 2008).
- Respiratory syncytial virus (RSV), which is a common and highly contagious respiratory virus that infects airway epithelial cells and other structural and resident immune cells, inducing a strong pro-inflammatory response which promotes virus clearance but can also be detrimental to the host (Lotz and Peebles 2012).

This thesis is composed of three chapters with the following concrete objectives:

1) To determine the extracellular anti-inflammatory effect of the synthetic surfactant based on recombinant human SP-C on the inflammatory response of LPS-stimulated mouse alveolar and peritoneal macrophages (chapter 1).

2) To evaluate the intracellular immunomodulatory action of synthetic surfactant vesicles internalized by alveolar macrophages on the activation state of these cells after being stimulated with LPS (chapter 2).

3) To investigate the intracellular immunoregulatory functions of internalized synthetic surfactant vesicles on the immune response of human alveolar epithelial cells (A549 cells) infected with human respiratory syncytial virus (chapter 3).

RESULTS AND CONCLUSIONS

❖ Chapter 1

To evaluate the extracellular anti-inflammatory effect of the synthetic surfactant on the inflammatory response of LPS-stimulated alveolar and peritoneal macrophages, we used two murine macrophage cell lines: MH-S AMs and RAW 264.7 peritoneal macrophages. Synthetic surfactant vesicles containing (sSPC) or not (sPL) human recombinant SP-C were added to the cells simultaneously with LPS.

The synthetic surfactant inhibited the pro-inflammatory response of LPS-stimulated AMs, disrupting the activation of the three key signaling pathways activated by LPS (the mitogen-activated protein kinase (MAPK) cascade, the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and nuclear factor-κB (NF-κB) activation). Consequently, the synthetic surfactant reduced the production of the pro-inflammatory mediators TNF-α and inducible nitric oxide synthase (iNOS) in AMs stimulated with LPS. This immunomodulatory action was not restricted to AMs, since sSPC synthetic surfactant was also shown to attenuate TNF-α secretion in peritoneal macrophages. Synthetic surfactant anti-inflammatory action decreased as surfactant vesicles were endocytosed by the cells through a clathrin-dependent mechanism. The results obtained also indicated that synthetic surfactant does not trap LPS molecules, suggesting that it acts directly on the surface of AMs, probably by interfering with LPS binding to its receptor.

Concerning the role of synthetic surfactant lipid and protein components in its immunomodulatory action, human recombinant SP-C is only involved in the reduction of iNOS mRNA production and protein levels, being synthetic surfactant lipid component mainly responsible for the disruption of LPS-induced pro-inflammatory response. Among the different lipid components, POPG was demonstrated to be essential for surfactant extracellular anti-inflammatory action, since lipid vesicles without POPG were unable to inhibit TNF-α release after LPS stimulation. Moreover, we proved that DPPC/POPG/PA

vesicles exerted a significantly higher inhibitory action on TNF-α release in LPS-stimulated AMs than DPPC/POPG vesicles. We also demonstrated that the presence of PA in DPPC/POPG/PA (sPL) vesicles increases ordered/disordered phase segregation in surfactant membranes, promoting the enrichment of POPG in disordered fluid domains and enhancing its anti-inflammatory action.

Conclusions: The results obtained indicated that synthetic surfactant lipid component, and specifically POPG, is responsible for the disruption of the pro-inflammatory response of LPS-stimulated alveolar macrophages. This effect is carried out in the extracellular medium, since it disappears as the vesicles are endocytosed by the cells. Considering that synthetic surfactant vesicles do not trap LPS molecules, their anti-inflammatory action seems to take place directly on the surface of alveolar macrophages, probably by interfering with LPS binding to its receptor; which explains the blockage exerted by the synthetic surfactant on all LPS-activated signaling pathways. On the other hand, recombinant human SP-C attenuated only LPS-elicited iNOS production. Our results also demonstrated for the first time that the presence of PA is essential for increasing ordered/disordered phase segregation in synthetic surfactant membranes, enriching POPG in disordered domains and thereby improving its immunoregulatory properties.

❖ Chapter 2

In order to analyze the potential intracellular immunomodulatory action of synthetic surfactant vesicles internalized by alveolar macrophages, the cells were preincubated with sSPC or sPL vesicles for 18 or 24 hours before being stimulated with LPS.

First of all, confocal microscopy analysis and flow cytometry studies revealed that synthetic surfactant small unilamellar vesicles are efficiently endocytosed by the cells after an 18- or 24-hour long incubation. However, sSPC and sPL multilamellar vesicles are only partially internalized during the same time period. For this reason, only synthetic surfactant small unilamellar vesicles were used to conduct this study.

On one hand, the results indicated that internalized synthetic surfactant lipids decreased TNF-α expression and secretion and IL-10 (anti-inflammatory cytokine) release in LPS-stimulated alveolar macrophages, being this effect very pronounced in the case of

TNF-α release (78 ± 3% inhibition). In contrast, endocytosed lipid vesicles also increased LPS-induced expression of four pro-inflammatory markers [interferon regulatory factor (IRF)1, C-X-C motif chemokines 10 and 11 (CXCL10 and CXCL11) and CD80] and of two mediators that can have pro- or anti-inflammatory activity depending on their environment [cyclooxygenase 2 (COX2) and suppressor of cytokine signaling 3 (SOCS 3)].

Concerning the signaling pathways that could be involved in these results, internalized lipid vesicles were proved to inhibit LPS-induced activation of NF-κB, as well as LPS-elicited Akt phosphorylation and activation. Moreover, they promoted glycogen synthase kinase 3 (GSK3) activation, probably as a consequence of the inhibition of Akt, which is a protein kinase that phosphorylates and inactivates GSK3. However, internalized lipid vesicles did not influence other LPS-activated signaling cascades, such as extracellular signal-regulated kinase (ERK) and p38 MAPKs, indicating that these signaling pathways are not involved in the immunomodulatory action of internalized synthetic surfactant lipids.

Experiments of GSK3 inhibition by lithium chloride demonstrated that the immunoregulatory action of internalized surfactant lipids is mediated at least in part by GSK3 activation, probably as a consequence of the inhibition of the PI3K/Akt pathway. Furthermore, the effect of internalized surfactant lipids on the reduction of LPS-induced TNF-α release could also be caused by their inhibitory action on NF-κB pathway activation.

Concerning the immunomodulatory role of synthetic surfactant protein component, the results indicate that human recombinant SP-C internalized by AMs specifically up-regulated the expression of two molecules involved in the development of alternative immune responses [IL-4 and CD200 receptor 3 (CD200R3)] as well as the production of the pro-inflammatory marker iNOS.

Conclusions: Once endocytosed by alveolar macrophages, both human recombinant SP-C and the lipid component present in synthetic surfactant membranes exert intracellular immunomodulatory actions, balancing the expression of pro- and anti-inflammatory mediators that modulate the response of the cells to LPS. The final result is the limitation of the pro-inflammatory response induced by the endotoxin. Our results also demonstrated for

the first time that the immunoregulatory effect of internalized surfactant lipids is mediated at least in part by GSK3 activation.

❖ Chapter 3

In order to examine the intracellular immunoregulatory functions of internalized synthetic surfactant on the immune response of A549 human alveolar epithelial cells infected with human RSV, the cells were preincubated with sPL or sSPC vesicles for 18 hours before being mock-infected or infected with the virus.

Analysis of SP-C protein levels in A549 AECs previously preincubated with sSPC for 18 hours confirmed synthetic surfactant internalization by these cells. Internalized synthetic surfactant vesicles were unable to inhibit viral replication in RSV-infected A549 AECs. Nevertheless, they decreased RSV-induced expression of two antiviral molecules [interferon-stimulated gene 15 (ISG15) and interferon-induced protein with tetratricopeptide repeats (IFIT) 1], of a protein that inhibits NF-κB and IRF3 transcription factors [tumor necrosis factor α-induced protein 3 (TNFAIP3)], and of two cellular receptors that recognize viral double stranded RNA [Toll-like receptor (TLR)3 and retinoic acid-inducible gene I (RIG-I)]. Synthetic surfactant also attenuated RSV-elicited activation of the PI3K/Akt pathway and ERK MAPK. However, internalized synthetic surfactant vesicles showed no modulatory effect on the activation of the NF-κB canonical pathway or p38 MAPK. Moreover, internalized surfactant lipids were found to be responsible for the down-regulation of ISG15 and RIG-I expressions and ERK activation in RSV-infected cells.

Conclusions: Our results provided evidence that internalized synthetic surfactant vesicles exert an intracellular anti-inflammatory action on RSV-infected A549 AECs, inhibiting RSV-induced expression of inflammatory mediators, as well as RSV-elicited activation of the PI3K/Akt pathway and ERK MAPK. Moreover, internalized synthetic surfactant lipid component has been demonstrated to be responsible for the down-regulation of ISG15 and RIG-I expressions and ERK activation in RSV-infected cells.

INTRODUCTION

1. RESPIRATORY SYSTEM PHYSIOLOGY

In the human body, cells require oxygen to accomplish aerobic metabolic processes. The carbon dioxide that results from these metabolic reactions is a waste product that must be eliminated from the body. The main function of the respiratory system is to supply oxygen to and remove carbon dioxide from the blood (Michael 2011).

The respiratory system is commonly divided into two sections: the upper respiratory tract and the lower respiratory tract. The first one is composed of the nasal cavity, the pharynx and the larynx. Its primary function consists in filtering, warming and humidifying the inhaled air before it reaches the conducting airways of the lower respiratory tract, composed of the trachea, the bronchial-tree and the lungs (figure 1A) (Michael 2011).

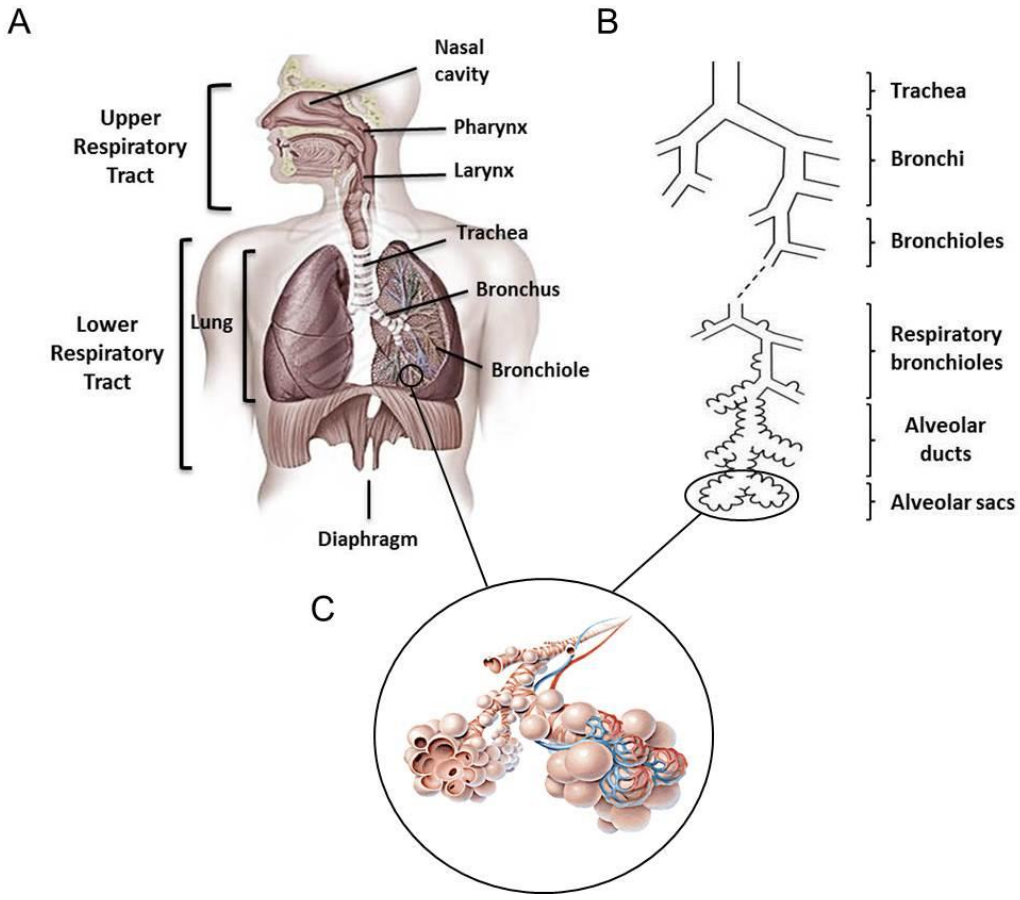


Figure 1: (A) Schematic representation of the upper and lower respiratory tracts. (B) Diagram of the bronchial-tree, based on the one of Weibel (Weibel 1984). (C) Schematic representation of two alveolar sacs and the capillaries lining the alveoli.

In the lower respiratory tract, the trachea divides into the right and left main bronchi. Each main bronchus divides into smaller bronchi, bronchioles and alveolar ducts, which terminate in semicircular alveolar sacs. Each alveolar sac is surrounded by four or more alveoli, which in turn are lined by blood capillaries (figures 1B and 1C) (Wang 2002; West 2012). The airways are divided 23 times from the trachea to the alveolar sacs (Weibel 1984). This branching structure of the lung is uniquely suited to maximize the surface area of the blood-gas barrier, which is necessary because gas exchange between the blood and the alveoli takes places by passive diffusion (Bates and Suki 2008).

2. ORGANIZATION OF THE ALVEOLAR WALL

The alveoli are the reservoirs in which gas exchange occurs: the oxygen enters from the atmosphere and diffuses into the blood, and the carbon dioxide enters from the blood and is exhaled to the air (Michael 2011). The number of alveoli in the adult human lung ranges from 300 to 500 million, and each one has an average diameter of 0.25 mm (Wang 2002; Daniels and Orgeig 2003; West 2012). This results in an alveolar surface area of 100 m^2, representing the largest surface of the body in contact with the outside environment. Approximately, 80% of the alveolar surface is lined with blood capillaries, which means that the area of the alveolar-capillary barrier is 80 m^2. Moreover, half of this barrier has a thickness of only 0.2 μm. This combination of large area and extreme thinness is optimal for gas diffusion (Weibel 1984).

The alveolar-capillary barrier is composed of three layers: the alveolar epithelium, the capillary endothelium, and a mixture of interstitial components that lies between them. Additionally, alveolar epithelial cells are covered by a thin layer of aqueous fluid lined by a lipid-protein complex called pulmonary surfactant. The alveolar fluid contains also immune cells, mainly resident alveolar macrophages (AMs) (figure 2) (West 2007; Michael 2011).

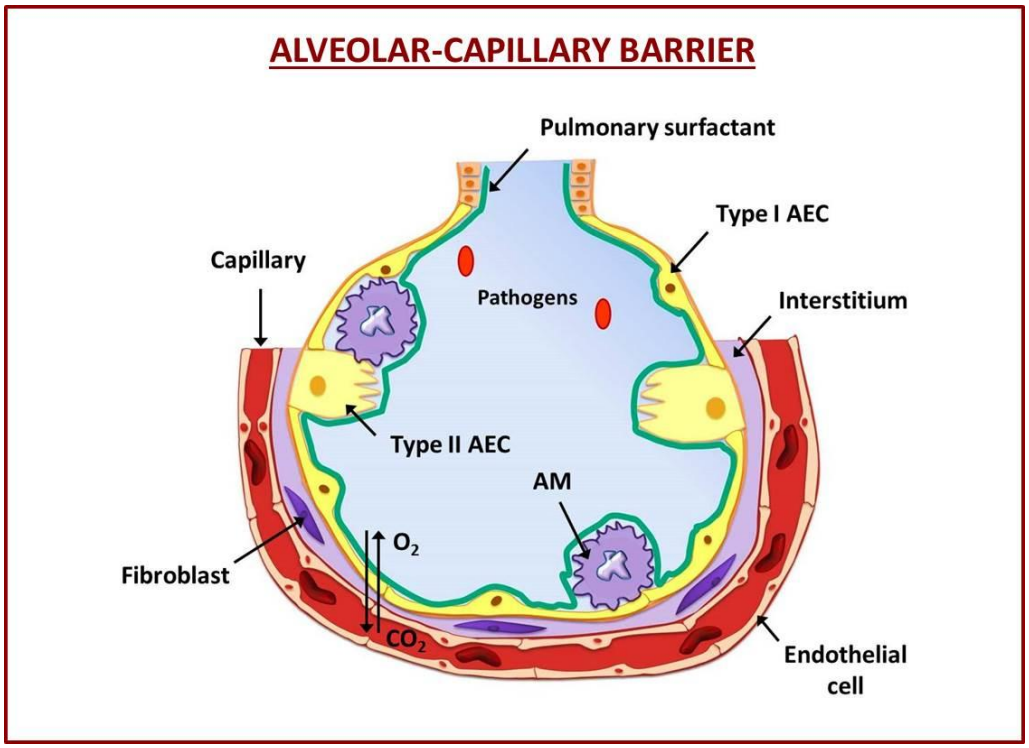


Figure 2: Schematic representation of the alveolar-capillary barrier. AEC: alveolar epithelial cell; AM: alveolar macrophage.

2.1 The alveolar epithelium

The alveolar epithelium is formed by two different cells: type I and type II alveolar epithelial cells (AECs) (Herzog et al. 2008).

2.1.1 Type I alveolar epithelial cells

Type I AECs are squamous epithelial cells with a central flattened nucleus and a thin peripheral cytoplasm that reaches 50 μm in diameter. They constitute 40% of alveolar lining cells but cover ~90% of the alveolar surface (Meyrick and Reid 1970; Wang 2002). These morphological features facilitate gas exchange.

Studies made with injured mature rodent lungs suggest that adult type I AECs are derived from type II AECs during alveolar repair; however it is still not known how this cell population is regenerated in human healthy lungs (Evans and Hackney 1972; Herzog et al. 2008).

In addition to that, type I AECs have limited numbers of mitochondria, but they express highly selective and non-selective cation channels, the cystic fibrosis transmembrane regulator and caveolins. These data support the concept that these cells may play an active role in the maintenance of lung homeostasis and the regulation of lung ion and fluid balance (Johnson et al. 2006; Herzog et al. 2008).

2.1.2 Type II alveolar epithelial cells

Type II cells are cuboidal cells with a diameter up to 15 μm. They are characterized by the presence of apical surface microvilli, a large basal nucleus with a prominent nucleolus, abundant cytoplasm with mitochondria, well-developed endoplasmic reticulum and Golgi apparatus and surfactant-containing lamellar bodies. They are generally located in the corner of the alveolus. They account for 60% of alveolar lining cells but cover only 5-10% of the alveolar surface (Meyrick and Reid 1970; Wang 2002; Herzog et al. 2008).

Type II AECs perform different functions essential to maintain alveolar homeostasis. Firstly, they are responsible for the synthesis, secretion and recycling of pulmonary surfactant. They also keep the alveolar space relatively free of fluid and regulate alveolar ion balance. Furthermore, they are able to proliferate or differentiate and replace injured type I AECs, being considered the stem cell of the adult alveolar epithelium. Finally, they participate in epithelial repair and play an important role in the immune defense of the lungs (Adamson and Bowden 1974; Fehrenbach 2001; Herzog et al. 2008).

2.2 The alveolar endothelium

The alveolar endothelium is the largest and most dense vascular bed in the human body (Wang 2002). It forms a continuous non-fenestrated barrier between the blood and the tissue. It is composed of highly attenuated endothelial cells resting on a thin basement membrane. The areas facing type I AECs are extremely thin: the luminal and abluminal plasma membranes are separated by a small amount of cytoplasm devoid of membrane invaginations and organelles. These areas are considered to be directly involved in gas exchange. As in other organs, endothelial cells also participate in different processes such as vascular permeability, coagulation and anti-coagulation, regulation of vascular tone and the immune host defense (Stan 2009).

2.3 The alveolar extracellular matrix

The extracellular matrix of the alveolar wall consists of two principal components: a basement membrane underlying alveolar epithelial and endothelial cells and an interstitial matrix maintained by fibroblasts (Burns et al. 2003).

Some areas of the alveolar-capillary membrane are extremely thin. There, the alveolar epithelium and endothelium are joined through a shared basement membrane. In other zones, the alveolar epithelial and capillary endothelial basement membranes are separated by a variable mixture of interstitial components and cells (Weibel 1984; Burns et al. 2003).

Basement membranes are composed of proteoglycans, fibronectin, entactin, laminin and type IV collagen. The interstitial matrix is comprised of collagen and elastin fibers embedded in a hydrated porous proteoglycan gel. Collagen provides tensile resistance while elastin assures tissue elasticity and recoil. The extracellular matrix also contains fibroblasts, mast cells, pericytes and interstitial macrophages (Weibel 1984; Burns et al. 2003; Dunsmore 2008).

3. PULMONARY SURFACTANT: COMPOSITION, METABOLISM AND BIOPHYSICAL FUNCTIONS

As mentioned before, epithelial cells and AMs are covered by a thin layer of aqueous fluid that contains pulmonary surfactant membranes (Michael 2011; Casals and Cañadas 2012).

Pulmonary surfactant is a membrane-based lipid-protein complex. Once secreted into the alveolar space by type II AECs, it forms an extracellular membrane network that efficiently covers the whole respiratory surface (Pérez-Gil 2008).

Its main function is to decrease the work of breathing and to reduce the surface tension at the air-liquid interface, thereby preventing the alveoli from collapsing at the end of expiration (Zuo et al. 2008; Casals and Cañadas 2012). Moreover, lung surfactant improves the mucociliary transport, prevents the formation of edema, and together with AMs, constitutes the front line of defense against inhaled pathogens and toxins (Griese 1999; Chroneos et al. 2010; Glasser and Mallampalli 2012).

Pulmonary surfactant is physiologically essential, and its lack, deficiency or dysfunction leads to the development of severe pulmonary diseases (Griese 1999; Glasser

et al. 2010; Gower and Nogee 2011). The first pathology that was linked to surfactant deficiency was the neonatal respiratory distress syndrome (RDS) (Avery and Mead 1959), a pathology in which the lungs of preterm newborns are unable to synthesize sufficient amounts of lung surfactant due to immaturity (Gower and Nogee 2011).

Since then, pulmonary surfactant has been the subject of extensive research that has helped the development of exogenous surfactant therapies, currently used to treat infants with neonatal RDS, meconium aspiration syndrome or respiratory failure from pneumonia (Willson and Notter 2011).

3.1 Pulmonary surfactant composition

The composition of lung surfactant is highly conserved among the different mammalian species studied. It contains approximately 90% of lipids and 10% of proteins by weight (figure 3) (Shelley et al. 1984; Veldhuizen et al. 1998; Postle et al. 2001).

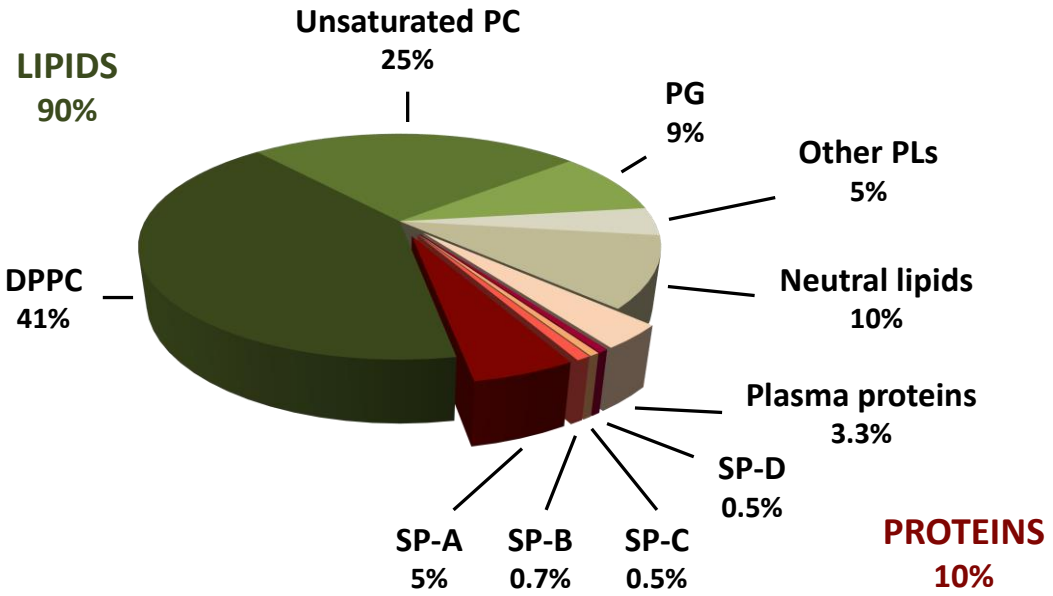


Figure 3: Diagram of pulmonary surfactant composition in percentages by weight. DPPC: dipalmitoylphosphatidylcholine; PC: phosphatidylcholine; PG: phosphatidylglycerol; PLs: phospholipids; SP-A, -B, -C, -D: surfactant proteins A, B, C and D.

3.1.1 Lipid composition

The lipid fraction of pulmonary surfactant consists mainly of phospholipids (~90-95% by weight); but it also contains a small amount of neutral lipids (~5-10% by weight), primarily cholesterol (Goerke 1998; Veldhuizen et al. 1998; Zuo et al. 2008).

Phospholipids are amphipathic molecules formed by a polar head and two hydrophobic acid chains. In aqueous phases, phospholipids usually organize themselves in the form of bilayers. At the alveolar air-liquid interface, surfactant phospholipids form an orientated monolayer, with their polar heads facing the water and the hydrophobic acid chains pointing toward the air. This monolayer excludes water molecules from the interface, lowering the surface tension at the end of expiration (see section 3.3.1) (Veldhuizen et al. 1998; Pérez-Gil 2008; Zuo et al. 2008; Casals and Cañadas 2012).

Phosphatidylcholine (PC) is the most prevalent class among surfactant phospholipids (~80% by weight of total phospholipids). Dipalmitoylphosphatidylcholine (DPPC) (16:0/16:0-PC) represents almost half of total phospholipids by mass and is the most abundant phospholipid molecular species in human lung surfactant (Veldhuizen et al. 1998; Bernhard et al. 2001; Postle et al. 2001). This saturated phospholipid can be packed to a very high density at the air-liquid interface in order to reduce alveolar surface tension and stabilize the lungs at the end of expiration (Casals and Cañadas 2012). The rest of PC molecular species are unsaturated, with monoenoic and dienoic fatty acids sterifying the *sn*-2 position of the glycerol backbone. In fact, palmitoyloleoylphosphatidylcholine (POPC) (16:0/18:1-PC) is the main unsaturated phospholipid in surfactant (Pérez-Gil 2008).

Pulmonary surfactant also contains anionic phospholipids: phosphatidylglycerol (PG) and phosphatidylinositol (PI). They are generally unsaturated and account for 8-15% by weight of surfactant phospholipid pool (Veldhuizen et al. 1998; Wright et al. 2000; Schmidt et al. 2002). Interestingly, the concentration of these anionic phospholipids in the lung is unusually high when compared with that of other tissues (Blanco and Pérez-Gil 2007; van Meer et al. 2008; Kandasamy et al. 2011). Palmitoyloleoylphosphatidylglycerol (POPG) (16:0/18:1-PG) is the dominant molecular species among surfactant PG (Wright et al. 2000).

Finally, other phospholipids, glycerides, free fatty acids, lysophospholipids, sphingolipids, and glycolipids are also present in pulmonary surfactant membranes at very low levels (Veldhuizen et al. 1998).

3.1.2 Protein composition

Apart from the lipid components, pulmonary surfactant also contains four specific surfactant-associated proteins named according to their chronological discovery as surfactant proteins A, B, C and D (SP-A, SP-B, SP-C and SP-D, respectively) (Possmayer 1988). They can be divided into two groups: SP-A and SP-D are large hydrophilic proteins, whereas SP-B and SP-C are small hydrophobic polypeptides (Serrano and Pérez-Gil 2006).

Furthermore, other nonspecific proteins are commonly isolated with pulmonary surfactant, including albumin, serum lipoproteins, immunoglobulins, growth factors or cytokines (Hawgood 1997).

SP-A and SP-D surfactant collectins

Hydrophilic SP-A and SP-D proteins belong to the non-serum mammalian collectins, which are secreted to mucosal surfaces or to the alveolar fluid (Wright 2005). Collectins, or calcium-dependent (C-type) lectins, are characterized by an N-terminal collagen-like domain and a globular C-terminal domain that contains a C-type carbohydrate recognition domain (CRD). Collectins are able to bind to a diverse array of sugar residues enriched in microbial surfaces in a calcium-dependent way, neutralizing a broad spectrum of foreign pathogens. Therefore, they are considered to play an essential role in the innate immune system (Lu et al. 2002).

- **SP-A**

SP-A represents approximately ~3-5% of the total mass of surfactant (Hawgood 1997). This protein has lipid-binding activity, and therefore is tightly associated with surfactant membranes. This ability of SP-A to interact with lipids is very important in different aspects of surfactant biology (Casals 2001).

Mammalian mature SP-A consists of 18 subunits assembled into a complex oligomeric structure that resembles a flower bouquet (figure 4). Its primary structure is highly

conserved among mammals (Casals 2001; Casals and García-Verdugo 2005; Wright 2005). SP-A oligomerization is an intracellular process that can be conceptualized in two parts. Firstly, SP-A trimers are built up by the association of three polypeptide chains. The collagen regions intertwine to form a collagen triple helix. Secondly, octadecamers are formed by lateral association of the N-terminal half of six triple-helical stems (figure 4) (Voss et al. 1988; Haas et al. 1991). Furthermore, mammalian SP-A is not only assembled in supratrimeric oligomers but can also form multimers by self-association in the presence of Ca^{2+} (Casals and García-Verdugo 2005).

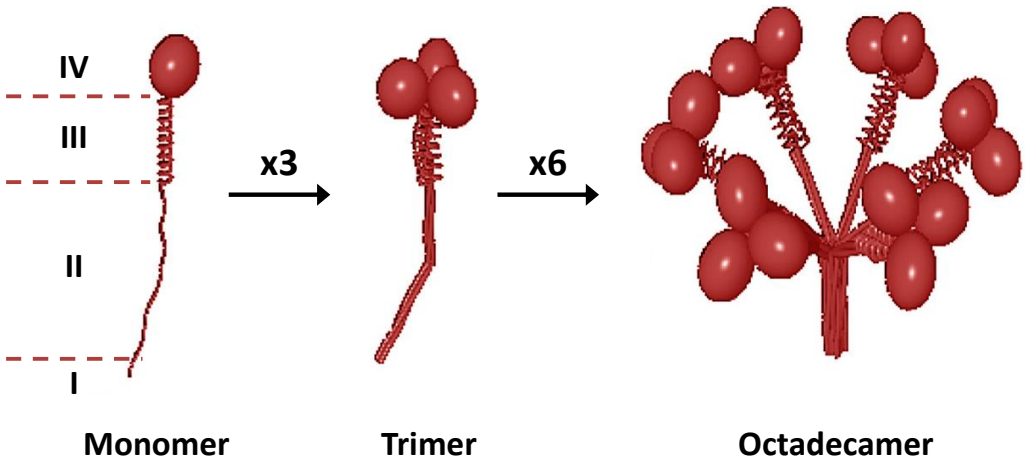


Figure 4: Models of monomeric, trimeric and oligomeric forms of SP-A. The four domains that constitute each SP-A monomer have been depicted: I) the N-terminal segment, II) the collagen-like domain, III) the neck region and IV) the C-terminal globular domain. Figure modified from (Casals and García-Verdugo 2005).

Each monomer (28-36 kDa) is composed of four structural domains (Casals 2001; Casals and García-Verdugo 2005; Wright 2005):

I) An N-terminal segment formed by 7-10 residues, involved in the establishment of intermolecular disulfide bonds that stabilize SP-A trimers and in covalent interactions between triple-helix stems to form oligomers.

II) A collagen-like domain of 79 residues characterized by 23 Gly-X-Y repeats with an interruption near the midpoint of the domain. This region presents

high tensile strength, stability and relative resistance to proteolysis that make it a perfect cross-linker between the C-terminal globular domain and the N-terminal segment. It is also required for the formation of supratrimeric oligomers and multimers. In addition to that, it functions as scaffolding that amplifies the ligand-binding activities of the CRD. Moreover, this domain is responsible for the binding of SP-A to different receptors expressed by AMs and AECs.

III) A neck region between the collagen-like and globular domains formed by a 35 aminoacid segment with high α-helical propensity.

IV) A C-terminal globular domain of 115 residues which participates in lipid-binding and in calcium-dependent binding of oligosaccharides. This region contains a glycosylation site (Asn^{187}) and two conserved tryptophan residues at positions 191 and 213. Additionally, four conserved cysteines form two intramolecular disulfide loops. Moreover, 18 aminoacid residues are highly conserved and common to the C-type lectins (Drickamer 1999). The three-dimensional structure of rat SP-A trimers composed of the C-terminal and neck domains has been determined. The basic structure of the globular domains consists of a structural core made up of 3 α-helices and 11 β-sheet strands (Head et al. 2003).

In humans and baboons, but not in other mammalian species studied, there are two functional genes encoding two different polypeptide chains, SP-A1 and SP-A2 (White et al. 1985; Floros et al. 1986; Gao et al. 1996). Both genes are expressed in type II AECs (Floros and Hoover 1998), but only SP-A2 gene is expressed in tracheal and bronchial submucosal gland cells (Goss et al. 1998; Saitoh et al. 1998). Indeed, it has been proposed that both gene products may be expressed in a 2:1 ratio (SP-A1:SP-A2) and associated through their collagenous domains to form heterotrimers (Voss et al. 1991).

After being translated, SP-A is modified in the rough endoplasmic reticulum and Golgi apparatus (cleavage of the signal peptide, proline hydroxylation and N-linked glycosylation) (McCormack 1998).

Supratrimeric oligomerization and multimerization of SP-A are required for many of its functions, because the binding activity of a single SP-A lectin domain is very low (Casals and García-Verdugo 2005; Sánchez-Barbero et al. 2005; Sánchez-Barbero et al. 2007). Among carbohydrates, SP-A binds preferentially to mannose and fucose, which are sugars commonly found on fungal and microbial surfaces (Haagsman et al. 1987). It also binds to lipids, preferentially to phospholipids whose headgroups are phosphocoline and that are esterified by long and saturated fatty acids, such as DPPC or sphingomyelin (King et al. 1986; Kuroki and Akino 1991; Casals et al. 1993). SP-A-lipid interaction has both hydrophobic and polar contributions (Casals 2001).

- **SP-D**

SP-D constitutes approximately ~0.5% of the total mass of surfactant, and in contrast to SP-A, is not associated with surfactant membranes (Hawgood 1997). Its solubility in the alveolar fluid is very high (Mason et al. 1998).

The structure of mature SP-D consists in 12 subunits assembled to produce a symmetric cruciform-shaped molecule (figure 5) (Crouch et al. 1994). SP-D oligomerization is an intracellular process, and thus the quaternary structure of the protein does not change after being secreted (Wallis and Drickamer 1999). Three subunits are assembled in trimers, and then, four trimers self-associate at their N-termini to form the dodecamers (figure 5) (Håkansson and Reid 2000). Moreover, human SP-D can also form higher-order multimers (Hartshorn et al. 1996).

Each monomer has a molecular weight of 43 kDa and contains four discrete structural domains (Crouch 2000; Håkansson and Reid 2000):

I) An N-terminal segment of 25 aminoacid residues, including two conserved cysteines at positions 15 and 20 (Crouch 2000; Håkansson and Reid 2000). These residues are responsible for the establishment of interchain disulfide crosslinks that stabilize the trimer and for the N-terminal association of four or more trimeric subunits (Crouch et al. 1994; Holmskov et al. 1995).

II) A collagen-like domain characterized by 59 Gly-X-Y repeats without interruptions (Håkansson and Reid 2000). This domain contributes to normal SP-D oligomeric assembly and secretion (Ogasawara and Voelker 1995;

Brown-Augsburger et al. 1996). Its length is highly conserved and determines the maximal spatial separation of trimeric C-terminal lectin domains within SP-D molecule (~100 nm), which may be essential for SP-D functions (Crouch 2000). Additionally, this region presents high tensile strength, stability and relative resistance to proteolysis that make it a perfect cross-linker between the C-terminal globular domain and the N-terminal segment (Håkansson and Reid 2000).

III) A neck region with an α-helical coiled-coil structure (Håkansson et al. 1999).

IV) A C-terminal globular lectin domain whose structure is composed of two anti-parallel β-sheets (one four-stranded and the other one five-stranded) and two α-helices (Håkansson et al. 1999). It also contains two bound calcium ions, 18 conserved aminoacid residues, and four cysteines which establish two intrachain disulfide bonds that stabilize the tertiary structure (Håkansson and Reid 2000).

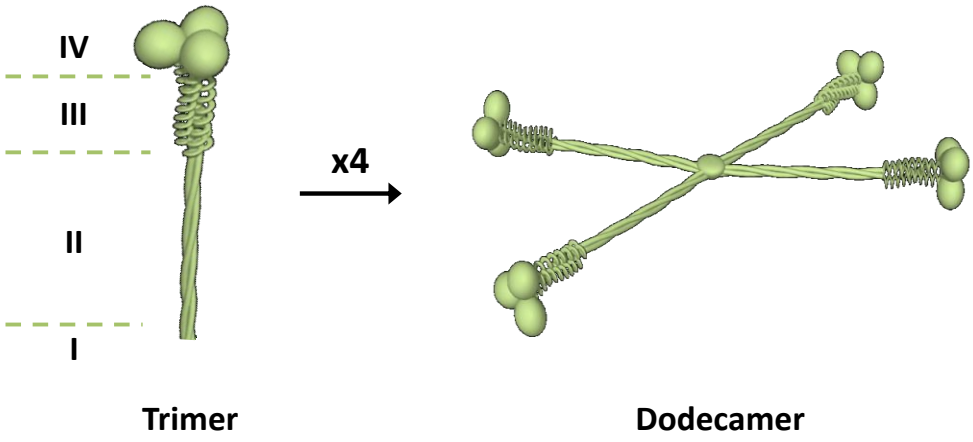


Figure 5: Models of trimeric and dodecameric forms of SP-D. The four domains that constitute each SP-D monomer have been depicted: I) the N-terminal segment, II) the collagen-like domain, III) the neck region and IV) the C-terminal globular domain.

Human SP-D is encoded by a single gene (Mason et al. 1998). SP-D folding and trimerization have been suggested to take place in the rough endoplasmic reticulum, whereas glycosylation would occur in the Golgi apparatus immediately prior to secretion (Crouch 1998).

SP-D lectin domain preferentially binds to simple and complex saccharides containing mannose, glucose or inositol (Persson et al. 1990; Lim et al. 1994). Additionally, it also interacts with PI (Ogasawara et al. 1992; Persson et al. 1992) and glucosylceramide (Kuroki et al. 1992). SP-D interaction with PI has both hydrophobic and polar contributions (Sano et al. 1998; Saitoh et al. 2000). A trimeric cluster of lectin domains is required for high-affinity binding to carbohydrates, because monomeric CRDs have 10-fold lower affinity than trimeric CRDs (Håkansson et al. 1999).

SP-B and SP-C hydrophobic polypeptides

SP-B and SP-C are small proteins strongly associated with surfactant lipids due to their high hydrophobicity (Blanco and Pérez-Gil 2007).

- **SP-B**

SP-B constitutes only 0.7% of the total mass of surfactant (Johansson and Curstedt 1997). However, its function is essential to maintain pulmonary homeostasis after birth.

SP-B is obtained from alveolar spaces as a covalent homodimer of 18 kDa composed of two 79 aminoacid polypeptides (Johansson and Curstedt 1997; Pérez-Gil 2008).

The three-dimensional structure of the full-length SP-B protein is currently not known (Casals and Cañadas 2012). Nevertheless, some studies have reported high-resolution structures of synthetic protein fragments (e.g., N-terminal 1-25 (Gordon et al. 2000) or 34-residue mini-B (Sarker et al. 2007)). Analysis of the sequence of SP-B reveals that this protein belongs to the family of the saposin-like proteins. Its structure might have a characteristic saposin fold with four amphipathic α-helices connected by unstructured loops (figure 6A) (Patthy 1991; Andersson et al. 1995). In addition to that, each monomer contains three intrachain disulfide bonds that link the highly conserved cysteine residues at positions 8 and 77, 11 and 71, and 35 and 46, respectively (Johansson et al. 1991b; Johansson and Curstedt 1997). These crosslinks may be responsible for the high

thermostability of the protein (Andersson et al. 1995), and at least one of them seems to be essential for SP-B proper function (Beck et al. 2000). The remaining cysteine residue at position 48 is involved in the establishment of an intermolecular disulfide bond to form SP-B dimers (Johansson et al. 1991b; Johansson et al. 1992), which may be also stabilized by hydrogen bonds (Zaltash et al. 2000).

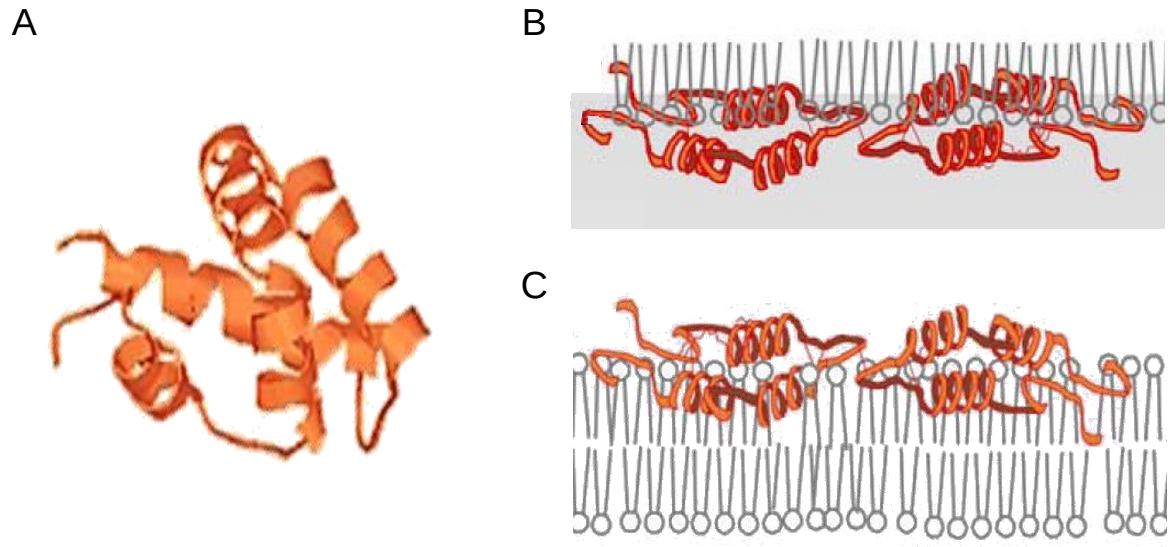


Figure 6: (A) Structural model of the monomeric form of SP-B based on predictive studies of the potential position of the helical segments in the protein, modified from reference (Casals and Cañadas 2012). (B) (C) Models of the disposition of SP-B dimers in lipid monolayers (B) and bilayers (C), modified from (Pérez-Gil 2008).

Human SP-B is encoded by a single gene (Pilot-Matias et al. 1989). This gene is first transcribed/translated into a 42 kDa preproprotein. This precursor is N-glycosylated on regions flanking the mature SP-B sequence, and processed to release the mature form of SP-B in the distal secretory pathway of type II AECs (Voorhout et al. 1992; Weaver et al. 1992; Hawgood et al. 1993; Hawgood et al. 1998). This pathway goes from the endoplasmic reticulum to the lamellar bodies (LBs), specialized organelles where surfactant lipids and SP-B and SP-C proteins are assembled and stored until secretion (Serrano and Pérez-Gil 2006).

SP-B is the only member of the family of saposin-like proteins permanently associated with membranes (Pérez-Gil 2008). The disposition of SP-B in surfactant membranes has not been demonstrated, but it is thought that SP-B interacts more or less peripherally with phospholipid membranes and monolayers, with the main axis of its amphipathic helical segments aligned in parallel to the plane of the lipid layer (figures 6B and 6C) (Vandenbussche et al. 1992b; Morrow et al. 1993a; Cruz et al. 1998; Cabré et al. 2012). Mature SP-B has a net charge of +7 (Hawgood et al. 1998), which promotes the interaction of the protein with surfactant anionic phospholipids, especially PG (Pérez-Gil et al. 1995). However, it is still not clear whether SP-B prefers PG- or PC-enriched membrane regions (Breitenstein et al. 2006; Seifert et al. 2007).

- **SP-C**

SP-C is the other surfactant small hydrophobic peptide. It constitutes only ~0.5% of the total mass of surfactant (Johansson and Curstedt 1997). Mature SP-C is composed of 35 aminoacid residues and has a molecular weight of 4.2 kDa (Curstedt et al. 1990; Johansson and Curstedt 1997). This protein is one of the most hydrophobic peptides known and is co-purified with SP-B and phospholipids in chloroformic extractions of pulmonary surfactant (Pérez-Gil 2008; Casals and Cañadas 2012).

The three-dimensional structure of mature SP-C has been determined by nuclear magnetic resonance (NMR) in chloroform/methanol solutions (Johansson et al. 1994). It consists of a very rigid and regular α-helix that covers approximately two thirds of the sequence, and a more polar unstructured N-terminal segment (figure 7A). Moreover, alignment of the aminoacid sequence of SP-C from seven animal species has revealed strictly conserved residues (Johansson et al. 1991a).

Firstly, the C-terminal α-helix comprises residues 9 to 34 and measures 3.7 nm. The stretch between positions 13 and 28 contains only aliphatic residues with branched side-chains, mainly valines. Notably, the leucine residue at position 22 is highly conserved. SP-C is positively charged due to the presence of the highly conserved dibasic pair Lys^{11}-Arg^{12} (Johansson et al. 1991a; Johansson et al. 1994). These positives charges are essential for SP-C functional properties (Creuwels et al. 1995).

Secondly, the N-terminal segment is formed by the eight first residues. The cysteines at positions 5 and 6 are palmitoylated via thioester bonds. Additionally, two prolines at positions 4 and 7 flank the two palmitoylated cysteines and are strictly conserved among species (Johansson et al. 1991a; Johansson et al. 1994).

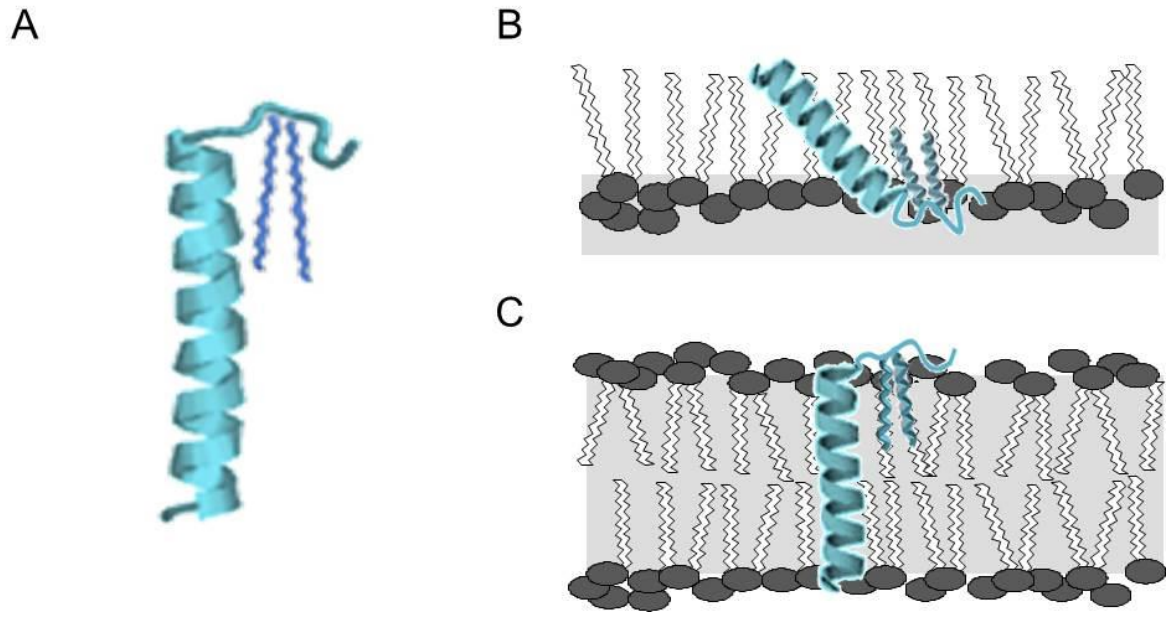


Figure 7: (A) Structural model of SP-C based on the conformation of the protein determined by NMR in organic solvents (Johansson et al. 1994). Figure modified from reference (Casals and Cañadas 2012). (B) (C) Models of the disposition of SP-C in lipid monolayers (B) and bilayers (C).

Unlike SP-B, SP-C is an integral membrane protein. The helical segment of mature SP-C adopts a transmembrane orientation in lipid bilayers, even if the protein is not palmitoylated (figure 7C) (Vandenbussche et al. 1992a). Its length is perfectly suited to traverse the thickness of a DPPC bilayer in a fluid state (see section 3.4.1). At an air-liquid interface *in vitro*, SP-C orientates with the C-terminal end exposed toward the air (figure 7B) (Gericke et al. 1997). However, it is still not known if SP-C is actually transferred into the alveolar air-liquid interface *in vivo* (Pérez-Gil 2008). Fluorescence measurements and NMR spectroscopy have revealed that the N-terminal segment and the positively charged

residues are located near the phospholipid headgroups (Horowitz et al. 1993; Morrow et al. 1993b). In fact, the study of synthetic peptides based on the sequence of SP-C N-terminal region has showed that they have a strong tendency to partition into the interface of phospholipid bilayers and monolayers even in the absence of palmitoylation (Plasencia et al. 2004; Plasencia et al. 2005). Additionally, some studies have suggested a possible dimerization of SP-C through its C-terminal domain in lipid bilayers or organic solvents, but the physiological relevance of these findings has still not been determined (Kairys et al. 2004; Luy et al. 2004).

Human SP-C is encoded by a single gene (Fisher et al. 1988; Glasser et al. 1988). SP-C is synthesized as a precursor of 197 aminoacids in which the mature sequence is flanked by N- and C-terminal propeptides. The sequence of the proprotein lacks consensus sequence for glycosylation or a typical signal peptide. Instead, the internal SP-C sequence acts as a signal peptide and directs SP-C proprotein to the membrane of the endoplasmic reticulum, where the proprotein orientates with the N-terminus in the cytosol and the C-terminal end in the lumen of the endoplasmic reticulum (Glasser et al. 1988; Keller et al. 1991). The lumenal C-terminal domain of SP-C proprotein is thought to stabilize the transmembrane domain preventing it from mysfolding and avoiding protein aggregation until the cysteines are palmitoylated (Gustafsson et al. 2001; Johansson et al. 2006; Johansson et al. 2009); while the N-terminal domain is required for the correct intracellular trafficking of SP-C (Kotorashvili et al. 2009). Subsequently, SP-C proprotein is palmitoylated in the Golgi apparatus and processed to the mature form of SP-C in the multivesicular body (MVB) and lamellar body (LB) compartments (Beers et al. 1994).

SP-C proprotein is internalized in lumenal vesicles in the MVB, and the incorporation of these vesicles into surfactant membranes is a SP-B-dependent process that allows SP-C to maintain its trans-membrane orientation throughout its biosynthesis (Conkright et al. 2010; Perez-Gil and Weaver 2010).

3.2 Pulmonary surfactant metabolism

Pulmonary surfactant is synthesized and secreted by type II AECs. The established model of surfactant metabolism envisages a cycle of three different steps: synthesis and storage, secretion, and reuptake for recycling and degradation (Goss et al. 2013).

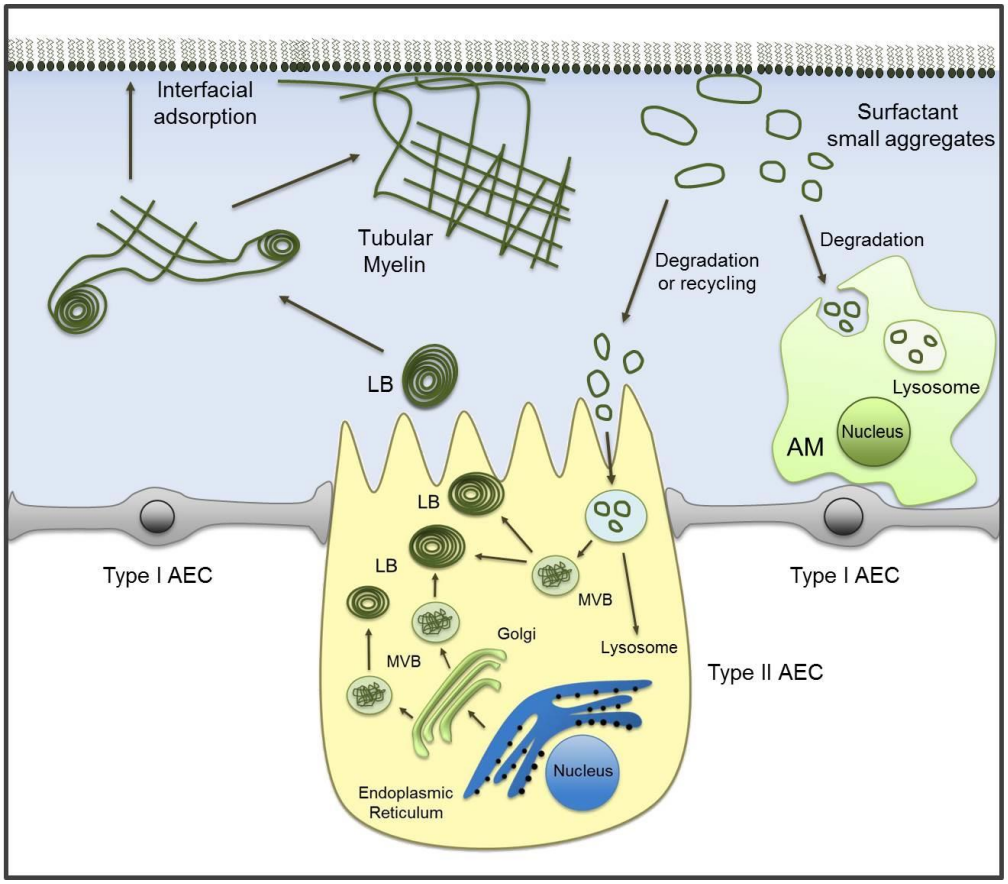


Figure 8: Schematic representation of pulmonary surfactant metabolism. AEC: alveolar epithelial cell; AM: alveolar macrophage; LB: lamellar body; MVB: multivesicular body.

3.2.1 Synthesis and storage

Surfactant lipids and hydrophobic proteins are assembled into complexes stored as tightly packed membranes in compartments called lamellar bodies (LBs) until secretion into the alveolar spaces (Perez-Gil and Weaver 2010). LBs are organelles that belong to the endosomal-lysosomal pathway (Weaver et al. 2002). They are characterized by an acidic interior containing lysosomal enzymes and proteins (Wasano and Hirakawa 1994; Yayoi et al. 2001). According to their morphology, two different types of LBs exist: some of them contain spherical concentric-like membranes with frequent contacts between them, and others accumulate tightly packed membrane stacks parallel to a main axis of the LB. These different morphologies could be the result of different packing stages (Pérez-Gil 2008).

Multivesicular bodies (MVBs) are thought to be the organelles which precede the LBs in the storage of surfactant components. They contain numerous internal unilamellar vesicles (Balis et al. 1985; Stahlman et al. 2000).

Figure 9: Transmission electron micrograph of a human type II AEC. Lamellar bodies (indicated as Lb) and typical apical surface microvilli are visible. Taken from (Fehrenbach 2001).

Maturation of LBs requires proper trafficking of surfactant components along the regulated exocytic pathway, which goes from the endoplasmic reticulum to the LBs (Weaver et al. 2002). As already explained, SP-B and SP-C are synthesized in the endoplasmic reticulum as large precursors that are processed while they are targeted via vesicular transport through the Golgi, the MVBs and the LBs (Johansson and Curstedt 1997; Perez-Gil and Weaver 2010). On the other hand, SP-A is not stored in LBs and is secreted through a different pathway, interacting with surfactant membranes once in the alveolar space (Wissel et al. 2001; Ochs et al. 2002). Phospholipids are also synthesized in the endoplasmic reticulum and can be transported to the LBs via three different pathways: vesicular transport, non-vesicular transport and diffusion at membrane contact sites (Perez-Gil and Weaver 2010).

Even though a study has reported anterograde vesicular transport of PC to the LBs (Chevalier and Collet 1972), it is widely assumed that phospholipids are carried to the LBs by specific phospholipid transfer proteins. They can also be transferred by passive diffusion at membrane contact sites, which would require accessory proteins to promote the formation of transient contact sites between organelles (Perez-Gil and Weaver 2010).

The ATP-binding cassette transporter A3 (ABCA3) is a transporter localized at the limiting membrane of LBs (Mulugeta et al. 2002). It uses the energy from ATP hydrolysis to pump surfactant lipids (mainly PC and PG) into endosomal-like compartments to generate LBs. Deficiency in ABCA3 results in neonatal lethal RDS, loss of mature LBs and decrease of PG and PC content in the lungs (Shulenin et al. 2004; Ban et al. 2007; Cheong et al. 2007).

On the other hand, cholesterol and minor phospholipids could be incorporated into surfactant membranes by ABCA3, by another unidentified transporter, or via the endocytic/recycling pathway (Perez-Gil and Weaver 2010).

Interestingly, SP-B is required for the correct maturation of LBs. Its lack causes lethal neonatal RDS accompanied by a deficit of LBs and an accumulation of MVBs in type II AECs (Clark et al. 1995; Stahlman et al. 2000; Wegner et al. 2007). It is thought that SP-B could promote the type of membrane-membrane interactions necessary for tight packing of membranes in the LBs (Pérez-Gil 2008).

3.2.2 Secretion

Pulmonary surfactant secretion into the alveolar space occurs via actin-dependent fusion of the limiting membrane of the LB with the apical plasma membrane of type II AECs, followed by extrusion of LB contents to the hypophase (figure 10A) (Agassandian and Mallampalli 2013; Goss et al. 2013).

Surfactant secretion is regulated by physiological and non-physiological agents (Agassandian and Mallampalli 2013). For example, labor and hyperventilation are important physiologic triggers for surfactant secretion *in vivo* (Rooney 2001; Dietl and Haller 2005). Extracellular ATP released from type I AECs (Mishra et al. 2011) or stress-induced calcium oscillations transmitted from type I to type II AECs via gap-junctions (Ashino et al. 2000) also stimulate surfactant secretion. Conversely, surfactant release can

also be down-regulated. For example, SP-A inhibits surfactant secretion by binding to a specific receptor on the surface of type II AECs (White and Strayer 2000).

Just after secretion, LB membranes are partially unpacked, probably due to re-hydration, pH neutralization, or decrease in Ca^{2+} concentration (figure 10A) (Pérez-Gil 2008). Nevertheless, a large fraction of secreted surfactant remains in the form of LB-like packed particles for a long time (minutes or hours). It has been demonstrated that individual secreted LBs are very efficient in transferring surfactant components into the air-liquid interface and that this process is modulated by changes in surface tension (Haller et al. 2004; Bertocchi et al. 2005).

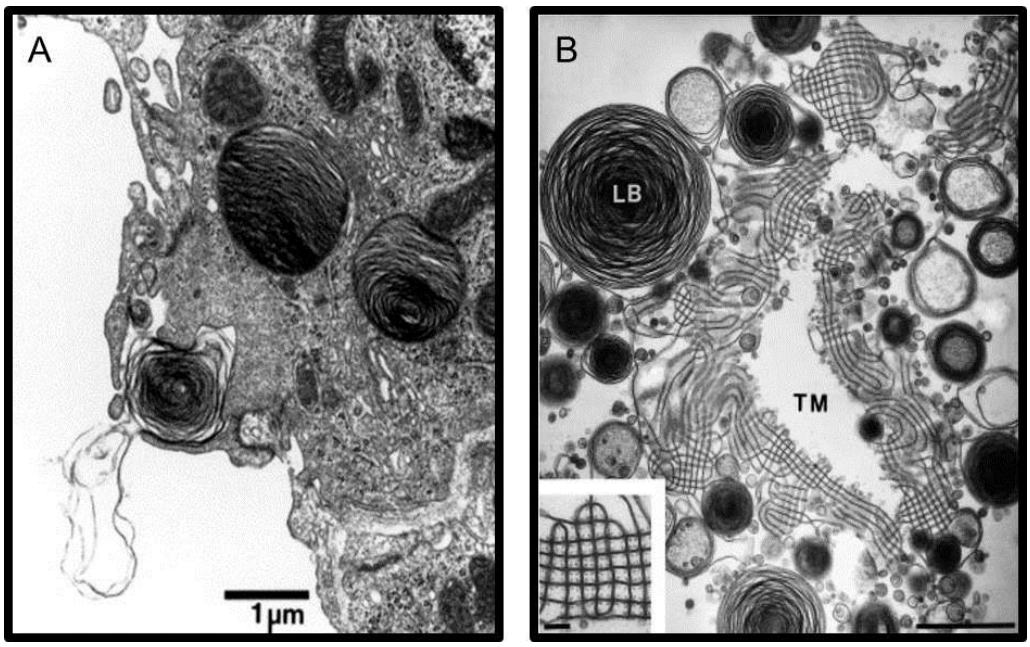


Figure 10: (A) Electron micrograph of the exocytosis of a LB from a type II AEC in an adult rat lung. Taken from (Rooney et al. 1994). (B) Electron micrograph section of a rat lung, showing lamellar bodies (LB) forming tubular myelin (TM) (bar = 1 µm). Inset: detail of tubular myelin presenting small projections in the corners, thought to represent SP-A (bar = 0.1 µm). Taken from (Goerke 1998).

However, secreted surfactant membranes often rearrange in a very unusual ordered network of membranes called tubular myelin (TM). This membrane organization has not been found in any other anatomic site (Pérez-Gil 2008). Its structure consists in very regular membrane square lattices, with the membranes apparently crossing or interacting at

regular distances (figure 10B) (Young et al. 1992). This organization requires the presence of at least DPPC, PG, SP-A, SP-B and Ca^{2+} (Suzuki et al. 1989). Even though membrane-membrane interactions could be promoted or stabilized by SP-B, the structure of TM is thought to rely on the properties of SP-A to interact with membranes and other surfactant proteins (Palaniyar et al. 2001; Pérez-Gil 2008). In fact, lack of SP-A expression results in loss of TM structures (Korfhagen et al. 1996). Surprisingly, TM is not essential for pulmonary homeostasis and lung function. Due to the high concentration of SP-A in these structures, it has been suggested that TM could play a primary antimicrobial role, collecting inhaled pathogens at the air-liquid interface (McCormack and Whitsett 2002).

Membrane unpacking finally results in the adsorption of surfactant lipid/protein complexes into the air-liquid interface, forming the surfactant monolayer. This promotes the reduction in surface tension required to stabilize the respiratory surface (Haller et al. 2004; Bertocchi et al. 2005).

3.2.3 Degradation and recycling

As stated above, surfactant is secreted as highly surface active large aggregates that adsorb rapidly into the air-liquid interface. However, compression and expansion cycles generate less surface active small aggregate vesicles (Veldhuizen et al. 1996). In addition to that, surfactant components can be oxidized as a result of repetitive exposure to air, or inactivated by materials inhaled or leaked from the capillaries (Rodríguez-Capote et al. 2006; Zuo et al. 2008; Perez-Gil and Weaver 2010). To maintain a fully functional surfactant, spent surfactant components must be removed from the alveolar space.

Surfactant can be cleared from the alveolar space by three different mechanisms:

1) Uptake and subsequent recycling or catabolism by type II AECs (Young et al. 1993).
2) Uptake and degradation by AMs (Yoshida and Whitsett 2004).
3) Transport from the alveolus up the bronchial tree (Bernhard et al. 2004).

Surfactant small aggregates are taken up by type II AECs by clathrin-dependent endocytosis accompanied by actin polymerization (Kalina and Socher 1990; Wissel et al. 2001; Jain et al. 2005). Subsequently, internalized surfactant can be recycled or degraded,

although the mechanism by which surfactant components are sorted for recycling or degradation is still not known (Agassandian and Mallampalli 2013). Recycled surfactant is carried by vesicular transport from the alveolar spaces to the LBs via the endocytic pathway, which converges with the biosynthetic pathway at the MVBs. The endocytic/recycling pathway is an important source of minor phospholipids and cholesterol (Perez-Gil and Weaver 2010). Interestingly, surfactant internalization is activated by the interaction of SP-A with its high affinity receptor on type II AECs (Wright et al. 1987). Moreover, SP-D binding to PI-rich surfactant complexes alters their structure and increases their uptake by type II AECs (Ikegami et al. 2005a; Ikegami et al. 2009).

The essential role played by AMs in the maintenance of surfactant homeostasis has been proved by their pharmacological depletion in adult rat lungs, resulting in a rapid increase of alveolar surfactant phospholipid pool (Forbes et al. 2007). Surfactant components internalized by AMs are largely degraded (Agassandian and Mallampalli 2013). In fact, mature AMs contain electron dense lamellar structures in their lysosomes (Zeligs et al. 1977). Recently, it was demonstrated that granulocyte macrophage colony stimulation factor (GM-CSF) stimulates peroxisome proliferator-activated receptor γ (PPARγ) transcription factor, which in turn activates the expression of the membrane lipid transporter ABCG1, which regulates surfactant catabolism (Baker et al. 2010; Malur et al. 2011). Surfactant degradation by AMs is enhanced by SP-A (Quintero et al. 2002). Furthermore, SP-D could influence surfactant catabolism through its ability to control the number of AMs (Aono et al. 2012).

Finally, surfactant can be cleared from the alveolar space by efflux up the bronchial tree. This process is facilitated by ciliary action in the larger airways, while in non-ciliated conducting bronchioles the transport depends on the physical force generated by the high surface pressure attained during compression of surfactant at expiration (Hohlfeld et al. 1997; Goss et al. 2013).

3.3 Surfactant biophysical functions

The main biophysical functions of pulmonary surfactant are: a) to reduce the surface tension are the air-liquid interface in order to prevent the alveoli from collapsing at the end of expiration; and b) to maintain the homeostasis of the alveolar-capillary fluid.

In addition to that, pulmonary surfactant components also participate in the immune defense of the lungs as anti-microbicidal and anti-inflammatory agents. This function will be described later (see section 4.2).

3.3.1 Reduction of the surface tension at the air-liquid interface

In the bulk of an aqueous phase, water molecules establish hydrogen bonds between them. These interactions are equilibrated in all space directions. However, at an air-liquid interface, the water molecules that are in direct contact with the air cannot interact with the more diluted gas molecules. This results in a net cohesive force that is directed toward the bulk of the aqueous phase and that tends to minimize the area of the interface (figure 11). Therefore, to maintain opened or to increase the area of the air-liquid interface requires a certain energy supply in order to compensate the interactions that water molecules at the interface cannot establish with the gaseous phase. This energy is called surface tension (γ). Taking into account that the area of the respiratory surface changes in every expansion and compression cycle, the lungs would have to exert a continuous effort to overcome the surface tension at the air-liquid interface.

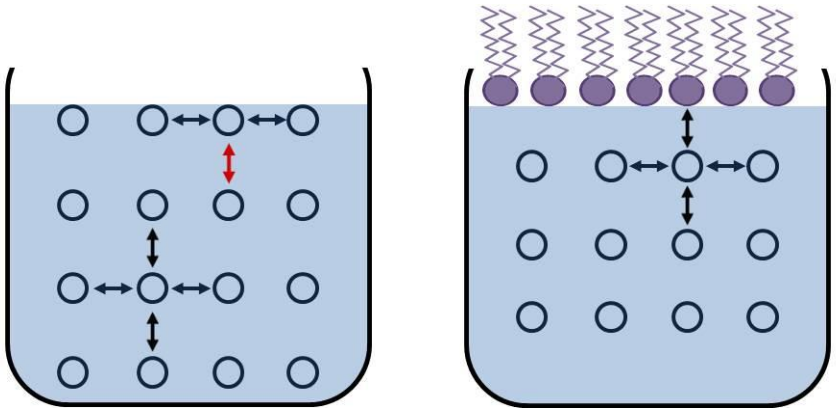


Figure 11: (Left) Schematic representation of the interactions that promote the surface tension. At an air-liquid interface, the water molecules that are in direct contact with the air cannot interact with the more diluted gas molecules. This results in a net cohesive force that is directed toward the bulk of the aqueous phase and that tends to minimize the area of the interface. (Right) Phospholipids form a stable monolayer at the air-liquid interface that excludes water molecules and decreases the unbalanced attractive forces between the interface and the bulk of the liquid, reducing the surface tension.

Pulmonary alveoli can be considered spherical cavities. The pressure inside an alveolus can therefore be defined by the law of Laplace:

$$P = 2\gamma/r \quad (1)$$

where P is the pressure inside the alveolus, γ is the surface tension and r is the radius of the alveolus. The law of Laplace establishes that P is directly proportional to γ and inversely proportional to r. Following this model, if two alveoli with different size were connected in the absence of surfactant, the smaller one would tend to collapse over the bigger one to balance the difference of pressure between them, especially at the end of expiration (figure 12) (Hills 1999; Notter 2000).

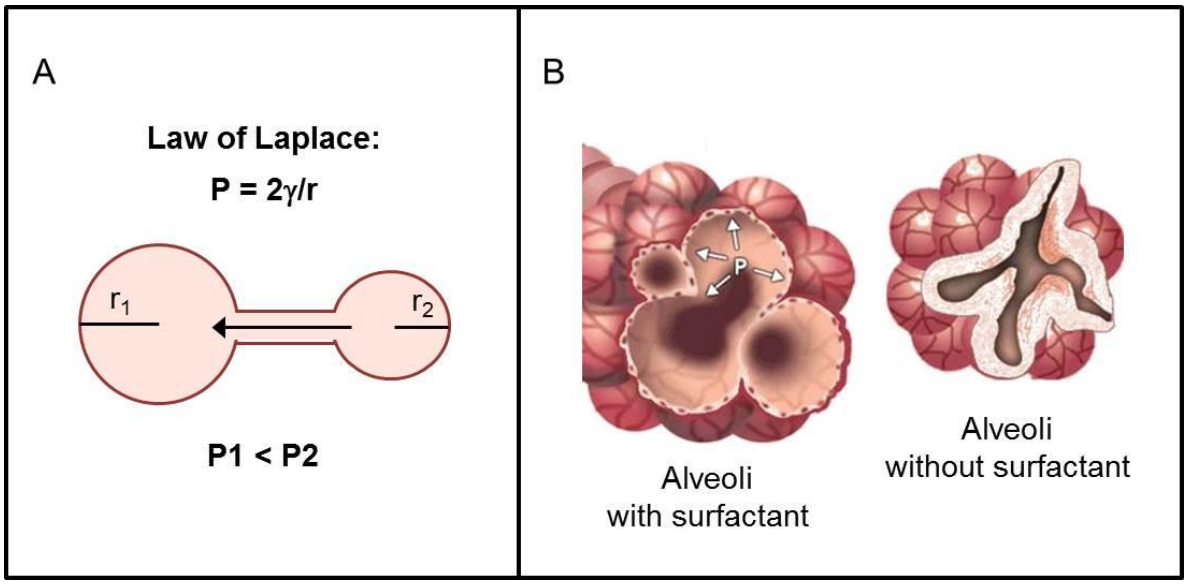


Figure 12: (A) Illustration of the law of Laplace. The small bubble tends to empty into the bigger one to balance the difference of pressure between them. (B) Representation of the state of alveoli in the presence or absence of surfactant.

Pulmonary surfactant components, especially surfactant phospholipids, tend to adsorb into the air-liquid interface forming a stable monolayer that excludes water molecules from the interface and decreases the unbalanced attractive forces between the interface and the bulk of the liquid, effectively lowering the surface tension of the interface (figure 11) (Notter 2000; Zuo et al. 2008). In fact, the surface tension of pure water at 37ºC is approximately 70 mN/m. However, during expiration, the surfactant film is highly

compressed, allowing the surface tension to reach values near 0 mN/m. Conversely, during inspiration the surfactant monolayer is expanded and the surface tension acquires a value of ~23 mN/m (Zuo et al. 2008). This dynamic variation of surface tension with area allows alveoli of different size to coexist stably during respiration (Notter 2000).

Surfactant deficiency or dysfunction results in alveolar collapse (atelectasis), uneven inflation and severe ventilation/perfusion alterations. These symptoms are commonly observed among infants with neonatal RDS (Notter 2000).

3.3.2 Maintenance of the alveolar-capillary fluid homeostasis

Surfactant prevents the development of pulmonary edema, which is defined as the abnormal accumulation of fluid in the lungs (Griese 1999; Chase and Wheeler 2007).

The net flow of fluid across the alveolar-capillary membrane and the driving forces that determine it are generally expressed using the Starling equation (Starling 1918):

$$Q = K_f[(P_c - P_i) - \sigma(\pi_c - \pi_i)] \quad (2)$$

where Q is the net flow across the alveolar-capillary membrane (from the capillary to the interstitial space); K_f is the filtration coefficient, which takes into account the contribution of the permeability and area of the alveolar-capillary membrane; P_c and P_i are the capillary and interstitial fluid hydrostatic pressures, respectively; π_c and π_i are the capillary and interstitial oncotic pressures, respectively; and σ represents the reflection coefficient of the alveolar-capillary membrane (values from 0 to 1, converging towards 0 when the membrane is extremely permeable to proteins and converging towards 1 when the membrane is impermeable to proteins) (Ganter et al. 2006; Chase and Wheeler 2007).

Under normal conditions, the balance of Starling forces favors a small flow of fluid and proteins out of the capillary endothelium into the interstitium. This fluid is efficiently drained by the lymphatics into the systemic venous system. When these forces are altered, fluid begins to accumulate in the interstitial tissue, and if the edema persists, it can even completely fill the alveolar space, severely impairing gas exchange (Ganter et al. 2006; Chase and Wheeler 2007).

Lung surfactant reduces the alveolar surface tension proportionally to the area of the alveoli and keeps the alveolar pressure and therefore the interstitial fluid hydrostatic pressure constant. When the pulmonary surfactant system is not functional, alveolar surface

tension increases. Subsequently, the value of P_i decreases and the net flow of fluid from the capillary toward the interstitium and as a consequence toward the alveolar space augments (Bredenberg et al. 1986).

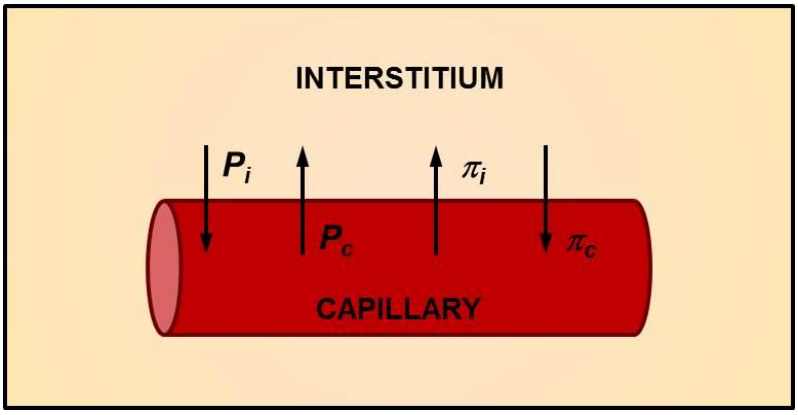


Figure 13: Diagram of the Starling forces that define the net flow of fluid across the alveolar-capillary membrane. P_c: capillary hydrostatic pressure; P_i: interstitial fluid hydrostatic pressure; π_c: capillary oncotic pressure; π_i: interstitial oncotic pressure. Based on the diagram from (Chase and Wheeler 2007).

3.4 Biophysical properties and organization of the surfactant film

3.4.1 Lateral phase segregation in surfactant bilayers and monolayers

As stated before, phospholipids dispersed in aqueous phases usually organize themselves in the form of closed bilayers. A characteristic property of lipid membranes is that they are able to undergo temperature-induced phase transitions. With increasing temperature, phospholipid bilayers "melt" from an ordered gel phase (L_β) to a disordered liquid-crystalline (L_α) phase. The temperature at which this transition takes place is called gel-to-liquid phase transition temperature or melting temperature (T_m) (Zuo et al. 2008; Casals and Cañadas 2012).

The two phases differ in the strength of the attractive Van der Waals interactions between adjacent lipid molecules (Stubbs 1983). In the gel phase, phospholipid fatty acid chains adopt an all-trans configuration that promotes strong interactions between phospholipid hydrophobic tails. Moreover, lipids are highly ordered and remain relatively immobile, without lateral or rotational mobility. If the phospholipid possesses saturated

chains, such as DPPC, the L_β phase is extremely packed. However, in the liquid-crystalline phase, also known as fluid-disordered phase, phospholipid molecules exhibit rotational and translational motions, conferring fluidity to the membrane; and phospholipid fatty acid chains undergo frequent trans-gauche isomerizations at their C-C bonds, increasing the area occupied by the chains and reducing lipid packing (McElhaney 1986; Pérez-Gil 2008; Zuo et al. 2008; Casals and Cañadas 2012).

Additionally, some phospholipids can form intermediate phases. DPPC, for example, exhibits a pretransition from the gel to the ripple phase ($P_{\beta'}$) and a very sharp transition from the ripple to the liquid-crystalline phase (Casals and Cañadas 2012).

The presence of cholesterol in lipid membranes alters this phase behavior. This neutral lipid exerts a fluidizing effect on lipids in the gel phase, because the intercalation of the planar steroid molecules decreases lipid packing. Meanwhile, cholesterol promotes the condensation of phospholipids in a fluid state, since it restricts the ability of the fatty acid chains to undergo trans-gauche isomerizations. Thus, the presence of cholesterol generates a new phase known as liquid-ordered phase (L_o) with properties intermediate between the L_α and L_β phases (Quinn and Wolf 2009; Mouritsen 2010; Casals and Cañadas 2012).

At air-liquid interfaces, phospholipids form an orientated monolayer with their polar heads facing the water and the hydrophobic acid chains pointing toward the air to avoid contact with water. Lipid packing in an interfacial monolayer is defined by temperature and changes in compression (Pérez-Gil 2008; Casals and Cañadas 2012). At low compression levels, phospholipids have low packing density and considerable configurational freedom, constituting the liquid expanded phase. Further compression of the monolayer gives rise to a highly packed state referred to as tilted condensed phase (MacDonald and Simon 1987; Nag and Keough 1993; Wüstneck et al. 2005; Casals and Cañadas 2012). This phase can only be reached at temperatures below the T_m of bilayers of the same composition (Casals and Cañadas 2012). The presence of cholesterol in an interfacial monolayer promotes the formation of liquid-ordered phases with an intermediate order between liquid expanded and tilted condensed phases (Worthman et al. 1997).

Giant unilamellar vesicles composed of native porcine lung surfactant membranes have been reported to display micrometer-sized fluid-ordered/fluid-disordered phase coexistence at physiological temperature (Bernardino de la Serna et al. 2004; Bernardino de

la Serna et al. 2009). The lack or reduced content of cholesterol gives rise to gel/fluid phase coexistence (Bernardino de la Serna et al. 2004; Sáenz et al. 2006; Sáenz et al. 2007). Furthermore, lateral phase segregation has also been observed in interfacial films formed by adsorption of LB-like particles secreted by rat type II AECs. These films spontaneously segregate into distinct liquid expanded, tilted condensed and liquid-ordered domains under near physiological conditions (Ravasio et al. 2010). This lipid lateral phase segregation is essential for pulmonary surfactant biophysical function (Casals and Cañadas 2012).

Surfactant proteins do not influence the lateral structure of surfactant membranes, but they segregate into different lipid domains. SP-B and SP-C have been shown to distribute preferentially into fluid-disordered regions of surfactant bilayers or into liquid expanded domains in surfactant interfacial films (Nag et al. 1997; Bernardino de la Serna et al. 2004). Conversely, SP-A interacts with liquid-ordered or gel-like regions of surfactant bilayers or condensed domains in surfactant monolayers (Casals et al. 1993; Worthman et al. 2000).

3.4.2 Biophysical properties of the pulmonary surfactant system

A functional pulmonary surfactant system must effectively accomplish three main processes essential for normal respiratory physiology: a) rapid adsorption at the air-liquid interface in order to form the surface active film, b) reduction of the surface tension to very low values during film compression at expiration, and c) film re-extension during expansion at inspiration (Serrano and Pérez-Gil 2006; Zuo et al. 2008).

After surfactant secretion as LB-like particles, surface active material is rapidly transferred to the air-liquid interface to form the surfactant monolayer (Haller et al. 2004; Bertocchi et al. 2005). This film is periodically compressed during exhalation (allowing the surface tension to reach values near 0 mN/m) and expanded during inhalation (increasing the surface tension up to ~23 mN/m) (Zuo et al. 2008). Nevertheless, different techniques have revealed that the interfacial film is thicker than a single monolayer. It consists of a surface monolayer and one or more lipid bilayers closely and functionally associated with the interfacial monolayer (Diemel et al. 2002; Bachofen et al. 2005; Follows et al. 2007).

Surfactant multilayers can be formed by multilamination and progressive adsorption of secreted LB-like particles (Ravasio et al. 2010). However, they also appear as a consequence of the monolayer-to-multilayer transition, which is a reversible and partial

monolayer collapse that takes place during surfactant film compression at expiration (Casals and Cañadas 2012). These bilayers remain closely attached to the monolayer, since they can readily re-spread to the monolayer during inspiration (Pérez-Gil 2008; Zuo et al. 2008). The cohesivity of surfactant monolayer-associated multilayers contributes to sustain the low surface tension reached at the end of exhalation, since it acts as a scaffold that helps the monolayer to resist such compression (Zuo et al. 2008; Casals and Cañadas 2012).

A competent pulmonary surfactant must be able to decrease the surface tension to near zero values at expiration with only a 20-30% reduction of the area. This can only be achieved by phospholipids that allow the formation of highly ordered and tightly packed solid-like films in a condensed phase. DPPC has a T_m of 41ºC and two saturated fatty acid chains, and it has been traditionally considered as the only surfactant component capable of reaching this low surface tension (Pérez-Gil 2008; Zuo et al. 2008).

Rapid adsorption, which is the bilayer to monolayer transformation, requires fluid phospholipid molecules with highly mobile structure (Zuo et al. 2008; Casals and Cañadas 2012). The slow adsorption of DPPC is compensated by the presence of unsaturated phospholipid species, which increase membrane fluidity, promote lateral phase segregation and decrease the T_m of surfactant films (Bernardino de la Serna et al. 2004; Zuo et al. 2008).

Interestingly, the presence of unsaturated phospholipids in the surfactant film does not hamper its ability to decrease surface tension upon compression. It has recently been demonstrated that compression-driven multilayers are enriched in unsaturated phospholipid species while saturated phospholipids are concentrated in the remaining interfacial monolayer. This is due to the fact that these structures start to form at the edge of solid-like tilted condensed phases or in fluid-like liquid expanded domains. The surface layer can therefore be highly compressed during expiration (Keating et al. 2012).

Moreover, SP-B and SP-C have been proved to promote rapid transfer of phospholipids into interfacial films (Oosterlaken-Dijksterhuis et al. 1991; Pérez-Gil et al. 1992; Ross et al. 2002). In the case of SP-C, its N-terminal segment interacts with and disturbs lipid packing in lipid membranes and films (Plasencia et al. 2004; Plasencia et al. 2005) different from the one where the α-helix is inserted (Serrano and Pérez-Gil 2006). Palmitoylation of this region of SP-C is thought to be essential to stabilize interdigitated-

like structures such as those present in intermediates of bilayer-to-monolayer conversion or bilayer-bilayer fusion (Plasencia et al. 2008). On the other hand, SP-B-mediated lipid adsorption may occur in a surface tension-dependent manner, promoted by the formation of a stalk-like structure between lipid bilayers and the monolayer. When the surface tension is below 23 mN/m, lipids are transferred from the monolayer to the vesicle. Conversely, when the surface tension is higher than this value, lipids are transferred from the bilayer to the monolayer (Baoukina and Tieleman 2011). Moreover, membrane perturbations induced by the amphipathic helical segments of SP-B could be important to facilitate surfactant lipid adsorption to the interfacial monolayer (Pérez-Gil 2008). Additionally, SP-A enhances adsorption and improves surfactant surface activity in the presence of SP-B and calcium (Venkitaraman et al. 1990; Schürch et al. 1992).

SP-B and SP-C also promote the formation and increase the cohesivity of surfactant multilayer structures associated with the interfacial film (Pérez-Gil 2008; Casals and Cañadas 2012). They are responsible for establishing the molecular connections between secreted surfactant particles and the surface film (Pérez-Gil 2008). Furthermore, they facilitate the folding of the surfactant monolayer during compression (Kramer et al. 2000; Krol et al. 2000). SP-C palmitoylation is essential to maintain SP-C containing membranes associated with highly compressed surfactant films at the end of expiration (Bi et al. 2002). SP-A also contributes to maintain the cohesivity of surfactant multilayers through its ability to bind simultaneously to different bilayers (Casals and García-Verdugo 2005).

Although the biophysical properties of the pulmonary surfactant system depend on surfactant phospholipids, SP-B and SP-C play an essential role in maintaining surfactant proper functionality. Induced depletion of SP-B in conditioned knock-out models has revealed that the lethal respiratory failure associated with the lack of this protein is not only due to the absence of a correct surfactant synthesis (Clark et al. 1995), but also to a lack of a proper dynamic flow of lipids interconnecting the whole alveolar surface (Melton et al. 2003). SP-C is not essential for life, as SP-B. Nonetheless, SP-C knock-out mice exhibit surfactant films with lower stability, and the absence of SP-C is associated with chronic respiratory failure (Glasser et al. 2001; Glasser et al. 2003; Gower and Nogee 2011).

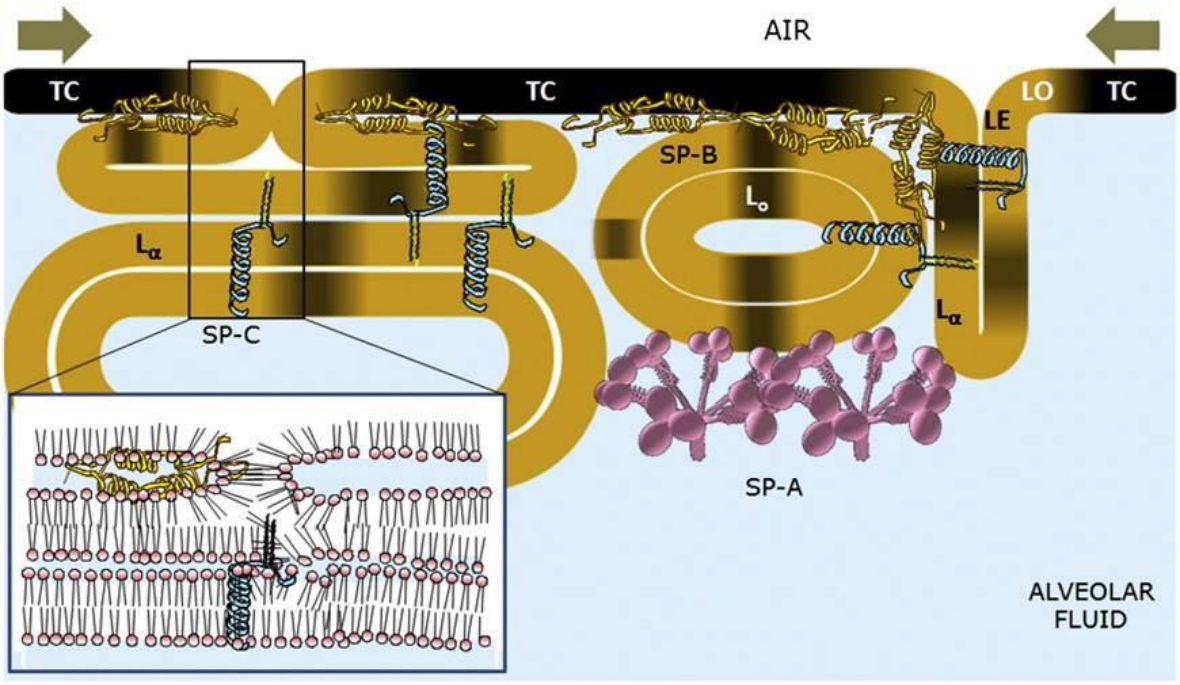


Figure 14: Schematic representation of the structure of multilayer surfactant interfacial films and the disposition of surfactant proteins SP-A, SP-B and SP-C. Tilted condensed, liquid expanded and liquid-ordered lipid phases in the surfactant monolayer are indicated as TC, LE and LO; while liquid-crystalline and liquid-ordered lipid phases in surfactant bilayers are indicated as L_α and L_o, respectively. SP-B and SP-C facilitate the formation of surfactant multilayer membranes associated with the interfacial film. The inset shows how SP-B and SP-C promote the establishment of membrane–membrane contacts that increase the cohesivity between surfactant layers. SP-A also contributes to maintain the cohesivity of surfactant membranes through its ability to bind simultaneously to different bilayers. Taken from (Casals and Cañadas 2012).

4. ROLE OF PULMONARY SURFACTANT IN THE INNATE IMMUNE SYSTEM OF THE LUNGS

The alveolar epithelium is the largest surface of the body in contact with the outside environment (Weibel 1984). It is continuously exposed to a diverse array of inhaled inorganic and organic particles, allergens and pathogens. Incoming antigens are principally highly immunogenic but harmless proteins of animals or plants. If these antigens evoked efficient immune responses, the lungs would suffer from chronic airway inflammation. The

immune system must therefore discriminate between this background antigenic noise and the much rarer harmful agents. Moreover, if an infection is established, the immune system must be able to kill and clear pathogens without triggering an excessive inflammatory response, which would damage alveolar tissues, compromising lung homeostasis and gas-exchange (Martin and Frevert 2005; Holt et al. 2008).

The immune system of the higher vertebrates can be divided into the innate and the adaptive immune systems. The first one is older, always active and immediately responsive. It consists of soluble proteins that neutralize microbial products and phagocytic leukocytes that migrate from the blood into the sites of inflammation or reside permanently in different tissues. The adaptive immune system is constituted mainly by lymphocytes that respond to signals of the innate immune system by producing high-affinity antibodies against specific peptide sequences present on the surface of pathogens. Cytokines and growth factors released by innate immune cells drive the specialized adaptive responses (Martin and Frevert 2005).

In the lungs, innate immune mechanisms are the first to face the array of microbial products that enters the lungs on a daily basis (Martin and Frevert 2005).

4.1 Description of the innate immune system in the alveolar space

As a consequence of inspiration, different particles can be inhaled. Large particles are carried into the conducting airways where they deposit under the influence of gravity. After that, they are eliminated by mucociliary clearance mechanisms and by soluble constituents of airway fluids (Martin and Frevert 2005; Glasser and Mallampalli 2012).

Particles smaller than 1 μm (such as bacteria and viruses) generally escape the defense mechanisms of the upper respiratory airways and are carried directly to the alveolar space, where they encounter the components of the alveolar innate immune system (Martin and Frevert 2005).

4.1.1 Soluble immune components

Soluble components present in the alveolar fluid participate actively in the clearance of invading pathogens from the alveolar space. Firstly, immunoglobulin G is the most abundant immunoglobulin in alveolar fluids. Together with complement proteins, they act

as opsonins which facilitate the phagocytosis of microbial agents by leukocytes (Martin and Frevert 2005).

Furthermore, alveolar fluids contain high concentration of lipopolysaccharide-binding protein (LBP) and soluble CD14 (Martin and Frevert 2005), which are two essential molecules for the recognition of bacterial lipopolysaccharide (LPS) by AMs (Schumann et al. 1990; Wright et al. 1990; Martin et al. 1992; Hailman et al. 1994). These proteins can diffuse from the plasma into the alveolar space (Martin and Frevert 2005). Nevertheless, LBP can also be synthesized by type II AECs (Dentener et al. 2000), and soluble CD14 is released from the plasma membrane of AMs by proteases (Senft et al. 2005).

Finally, surfactant components have been recognized to play an essential role in the immune defense mechanisms of the lungs, which will be described later in section 4.2 (Chroneos et al. 2010; Glasser and Mallampalli 2012).

4.1.2 Alveolar epithelial cells

AECs are considered to be important effector cells of the innate immune system of the lungs (Fehrenbach 2001). Firstly, they act as a physical barrier to exclude invading pathogens from the organism (Holt et al. 2008).

Secondly, type II AECs synthesize and secrete into the overlying fluid a wide range of regulatory and effector molecules involved in front-line defense against pathogens, including LBP (Dentener et al. 2000), complement proteins (Strunk et al. 1988), and surfactant components (Agassandian and Mallampalli 2013).

Moreover, type II AECs express a variety of pattern recognition receptors (PRRs) that recognize different pathogen-associated molecular patterns (PAMPs), including Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Armstrong et al. 2004; Liu et al. 2007). Thus, these cells can directly respond to pathogenic agents and initiate the innate immune response (Zhang et al. 2001; Unkel et al. 2012; Chuquimia et al. 2013). Interestingly, type I AECs have recently been demonstrated to be also able to mount innate immune responses during bacterial infections (Yamamoto et al. 2012).

Furthermore, type II AECs have the ability to modulate the response and the activity of other immune cells. In response to an infectious challenge, these cells can release a wide

range of cytokines, such as the pro-inflammatory interleukin (IL)-6, IL-8, and tumor necrosis factor α (TNF-α) (Thorley et al. 2011), and chemokines, including regulated on activation normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1) which chemotactically attract monocytes (Rosseau et al. 2000; Thorley et al. 2011). They also produce GM-CSF, which stimulates macrophage growth and induces the recruitment and activation of dendritic cells (Fehrenbach 2001; Unkel et al. 2012). Additionally, they have also been shown to regulate the activity of lung interstitial macrophages (Chuquimia et al. 2013). Finally, type II AECs can also promote T cell activation (Zissel et al. 2000).

4.1.3 Alveolar macrophages

Under normal conditions, AMs account for approximately 95% of airspace leukocytes, with 1-4% lymphocytes and only about 1% neutrophils. They are considered the sentinel phagocytic cells of the innate immune system of the lungs (Martin and Frevert 2005).

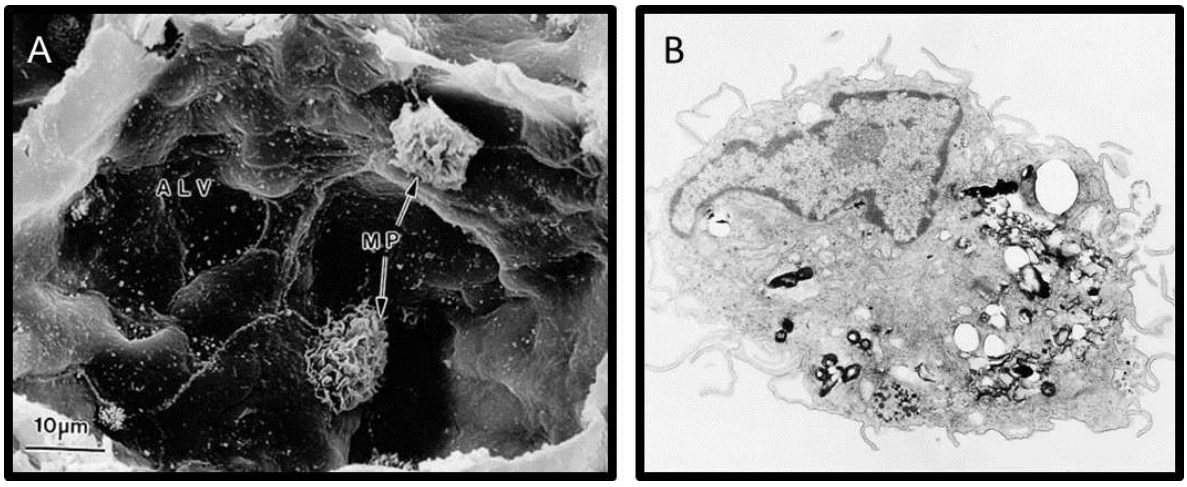


Figure 15: (A) Scanning electron micrograph of a rat lung showing two ruffled alveolar macrophages (indicated as MP) in the alveolar space (indicated as ALV). Taken from (Martin and Frevert 2005). (B) Electron micrograph of a human alveolar macrophage (magnification x 7056). Taken from (Keane et al. 1997).

Macrophages are specialized hematopoietic cells distributed throughout the different tissues of the body (Lambrecht 2006). Morphologic studies have revealed that resident

AMs are large, mature cells with an increased cytoplasm/nucleus ratio (figure 15B). They are often observed protruding from the alveolar epithelial walls into the lumen of the lungs (figure 15A) (Laskin et al. 2001).

AMs play a central role in the maintenance of the immunological homeostasis of the lungs (Holt et al. 2008). They are avidly phagocytic and ingest the inhaled particulates that reach the alveolar space without triggering an immune response (Martin and Frevert 2005). In fact, phagocytosis and sequestration of antigens protect local tissues from the development of inflammatory immune responses (MacLean et al. 1996). As a result, AM damage promotes an increase in the susceptibility of the host to bacterial infections and toxicants (Laskin et al. 2001).

Normally, AMs are kept in a quiescent state: they produce low levels of pro-inflammatory cytokines but they secrete high levels of the immunosuppressive cytokine IL-10 (Lambrecht 2006). They are not considered good antigen-presenting cells (Martin and Frevert 2005; Jakubzick et al. 2006). Furthermore, AMs have been proved to suppress the induction of adaptive immune responses. In macrophage depleted rodents, interstitial dendritic cells exhibit enhanced antigen-presenting function, indicating that AMs disrupt T-cell activation by modulating dendritic cell antigen-presenting function (Holt et al. 1993). They also regulate dendritic cell numbers, migration and localization, as proved by studies of AM depletion in mice (Jakubzick et al. 2006).

The atypical phenotype of AMs is largely influenced by lung environment (Gwyer Findlay and Hussell 2012). Surfactant components play a critical role in the regulation of AM phenotype and activity, as detailed in section 4.2 (Chroneos et al. 2010; Glasser and Mallampalli 2012). Moreover, the high levels of GM-CSF in the alveolar space favor an increase in the expression of CD11c by AMs, which may help their phagocytic function (Guth et al. 2009).

In fact, the functional phenotype of newly recruited monocytes in the lungs differs markedly from that of resident AMs. Monocytes have a pro-inflammatory phenotype and are efficient antigen-presenting cells that promote T-cell and dendritic cell activation. They gradually acquire the phenotype of resident AMs over a period of days (Bilyk and Holt 1995; Murray and Wynn 2011).

The particular characteristics of AMs are due also in part to their interaction with AECs. Firstly, resting AMs express CD200 receptor (CD200R), and its levels can be increased by different pulmonary pathogens. Its ligand is CD200, which is found in many cell types including type II AECs (Snelgrove et al. 2008; Goulding et al. 2011). The interaction between CD200R and CD200 elicits immunoregulatory events in AMs, which are the cells that express CD200R (Zhang et al. 2004). In fact, mice lacking CD200 have been proved to be more sensitive to influenza virus infection, with enhanced AM activity and delayed resolution of inflammation (Snelgrove et al. 2008). Secondly, another mechanism of AM function inhibition has been proposed. Resting AMs have been shown to adhere closely to type II AECs and to induce the expression of the αvβ6 integrin on these cells. This integrin activates transforming growth factor β (TGF-β), which in turn suppresses AM activation and cytokine production. Inhibition of macrophage activity through αvβ6-TGF-β interactions may be unique of the lungs (Lambrecht 2006).

However, AMs maintain their capacity to be activated by extrinsic or intrinsic stimuli (Holt et al. 2008). They express numerous PRRs including TLRs and RLRs. These receptors detect PAMPs, such as microbial nucleic acids, lipoproteins and carbohydrates, or damage-associated molecular patterns (DAMPs) released from injured cells. Activation of PRRs triggers their oligomerization and the initiation of intracellular signaling cascades that activate different transcription factors (such as nuclear factor-κB (NF-κB), cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB), CCAAT-enhancer box binding protein (c/EBP), interferon regulatory factors (IRFs), among others) and culminate with the expression of inflammatory mediators (reviewed in Newton and Dixit 2012).

For example, stimulation of TLRs in AMs leads to their detachment from type II AECs. Then, AMs promote the development of an inflammatory response. Notably, they release IL-8 and related CXC chemokines that recruit neutrophils from the lung capillaries. They also secrete MCP-1 and RANTES, which recruit activated pro-inflammatory monocytes and lymphocytes into the site of inflammation (Martin and Frevert 2005; Lambrecht 2006). Conversely, they also participate actively in the resolution of inflammation, clearing the remaining cellular debris and remodeling the lung parenchyma (Knapp et al. 2003; Alber et al. 2012).

The functions of AMs must be tightly controlled, since their dysregulation can make them become detrimental to the host due to epithelial cell death, septic spread of bacteria to the blood, excessive deposition of extracellular matrix components or dysregulated repair of damaged tissues. AMs have been demonstrated to play a critical role in the development of inflammatory lung diseases, idiopathic pulmonary fibrosis, secondary bacterial infections, allergy, asthma and chronic obstructive pulmonary disease among others. Therefore, the study of the mechanisms that regulate and control the activity and phenotype of AMs is currently the subject of extensive clinical and basic research (reviewed in Dasgupta and Keegan 2012; Gwyer Findlay and Hussell 2012; Pappas et al. 2013).

Macrophage functional subpopulations

Macrophages are highly heterogeneous cells that can be subcategorized into subpopulations based on their anatomical location and functional phenotype. Therefore, several macrophage subsets with distinct specific functions have been described (Murray and Wynn 2011).

The first category are classically activated macrophages, also known as M1 macrophages. This macrophage phenotype is induced by the recognition of PAMPs or DAMPs by PRRs, in synergy with natural killer cell-derived interferon γ (IFN-γ). These macrophages mediate host defense from a wide array of bacteria, protozoa and viruses, and promote anti-tumor immunity. They produce reactive oxygen and nitrogen species that facilitate pathogen killing. Moreover, they release pro-inflammatory cytokines such as TNF-α, IL-1, IL-12 and IL-23, and are drivers of antigen-specific type I helper T cell (T_H1 cell) and T_H17 cell responses. T_H1 cells in turn release more IFN-γ that expands the population of M1 macrophages and enhances their microbicidal and tumoricidal activity (Mosser and Edwards 2008; Galli et al. 2011; Krausgruber et al. 2011; Murray and Wynn 2011).

The second subpopulation is referred to as alternatively activated macrophages or M2 macrophages. M2 polarization is promoted by IL-4 and IL-13 which activate signal transducer and activator of transcription (STAT)6 transcription factor (Gordon and Taylor 2005; Galli et al. 2011). These cytokines are primarily produced by T_H2 cells, basophils, nuocytes and natural helper cells. IL-10, IL-21, IL-33 and GM-CSF also regulate M2

macrophage differentiation (Galli et al. 2011). Additionally, IL-4 activates the expression of Jmjd3, a demethylase of histone 3 lysine 27. This protein increases the expression of M2 markers, such as arginase I or chitinase 3-like-3 (Ym1) (Ishii et al. 2009). M2 macrophages antagonize toxic M1 responses, have anti-inflammatory functions and suppress anti-tumor immunity. Moreover, they facilitate wound repair, tissue remodeling and angiogenesis, regulate glucose metabolism, and mediate the expulsion of extracellular parasites from the gut (reviewed in Galli et al. 2011; Murray and Wynn 2011).

A third subpopulation has also recently been described, called regulatory macrophages. This macrophage phenotype is induced by TLR ligands together with immunoglobulin G complexes, apoptotic cells or prostaglandins. They secrete high levels of IL-10 and TGF-β1. Additionally, they tend to promote T_H2 or regulatory T cell responses. They suppress antimicrobial responses, chronic inflammation and anti-tumor immunity. Furthermore, IL-10 released by regulatory macrophages plays a critical role in constraining the pro-inflammatory response of newly recruited monocytes at the sites of inflammation, avoiding collateral tissue damage (Mosser and Edwards 2008; Galli et al. 2011; Murray and Wynn 2011).

Finally, tumor-associated macrophages and the monocytic subset of myeloid-derived suppressor cells actively suppress tumor immunity (Mosser and Edwards 2008; Galli et al. 2011; Murray and Wynn 2011).

Thus, macrophages adopt context-depending phenotypes that either promote or inhibit host antimicrobial defense, anti-tumor immunity and inflammation. It is widely accepted that macrophages represent a spectrum of activated phenotypes rather than separated subpopulations. They can switch from one phenotype to another in response to diverse signals present in their microenvironment. This plasticity represents a powerful mechanism to control and regulate innate immune responses avoiding tissue damage (Galli et al. 2011; Murray and Wynn 2011).

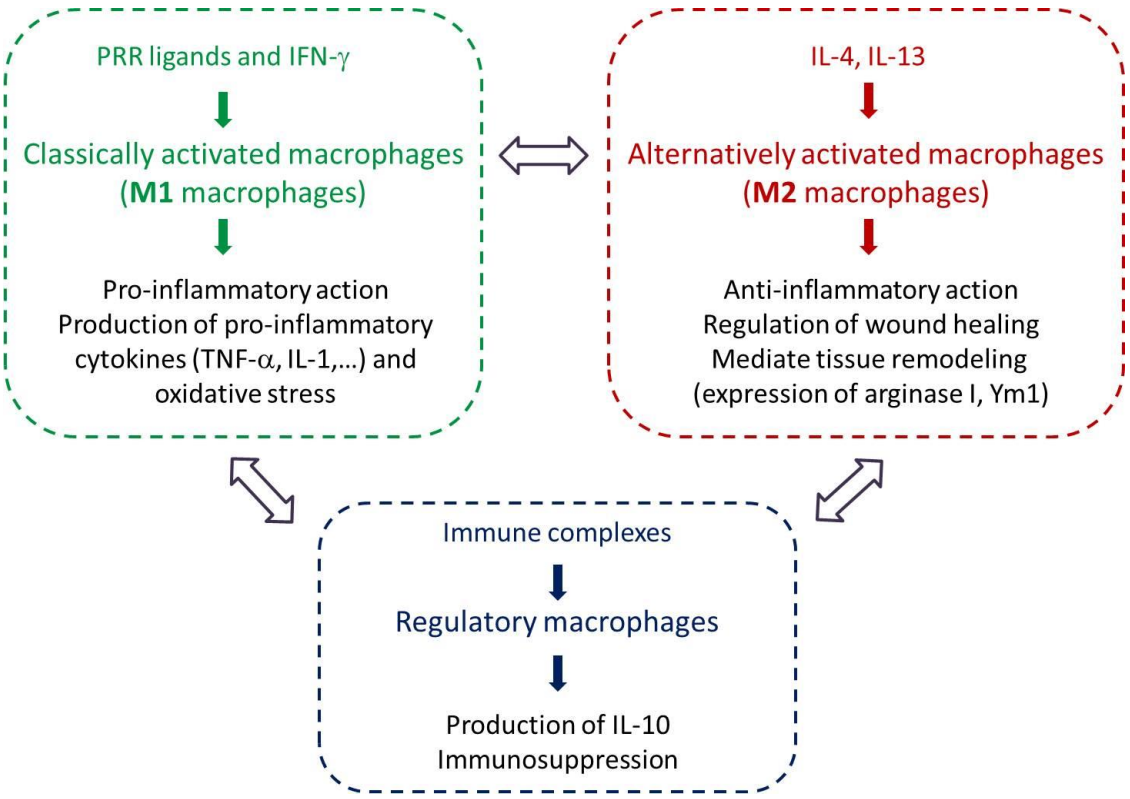


Figure 16: Diagram of the main macrophage subpopulations based on their functional phenotype. Rather than separated subsets, macrophages represent a spectrum of activated phenotypes. They can switch from one phenotype to another in response to diverse signals present in their microenvironment.

4.2 Role of surfactant components in host defense

Pulmonary surfactant is currently considered as an integral component of the lung's innate immune system. It helps to maintain sterility preventing microbial infections, to balance immune reactions and to control inflammatory responses in the distal airways (Chroneos et al. 2010).

Firstly, surfactant material acts as a physical barrier to pathogens (Wright 2005; Glasser and Mallampalli 2012). Moreover, surfactant lipids and proteins have been proved to accomplish different important immune-related functions (Chroneos et al. 2010; Glasser and Mallampalli 2012).

Extensive research conducted with genetically manipulated mice supports the concept that genetic polymorphisms in one or more surfactant protein genes are associated with enhanced susceptibility to pulmonary inflammatory disease in humans (Chroneos et al. 2010).

Furthermore, abnormal surfactant levels or composition have been linked with inflammation in acute lung injury (ALI)/acute RDS (ARDS), pulmonary fibrosis, emphysema, cystic fibrosis and chronic obstructive pulmonary disease, among others (Chroneos et al. 2010).

Understanding the mechanism by which surfactant components are able to regulate lung immune responses could help to develop new anti-inflammatory drugs to counteract pulmonary inflammatory diseases, especially in situations in which lung respiratory function is compromised.

4.2.1 Surfactant lipids

A significant and increasing body of data indicates that surfactant lipids play active roles in the innate immune host defense of the lungs through diverse mechanisms (Blanco and Pérez-Gil 2007; Chroneos et al. 2010; Glasser and Mallampalli 2012; Numata et al. 2012b).

Dipalmitoylphosphatidylcholine

As described before, DPPC is the most abundant phospholipid molecular species in surfactant monolayers (Bernhard et al. 2001), and is considered as primarily responsible for the surface tension reducing properties of surfactant (Perez-Gil and Weaver 2010). Nevertheless, DPPC has also been reported to participate in the regulation of immune responses in the lungs (Blanco and Pérez-Gil 2007; Chroneos et al. 2010).

Firstly, DPPC is able to diminish group B *Streptococcus* infection and subsequent IL-8 production in A549 lung epithelial cells. However, the mechanism underlying this effect has not been yet unraveled (Doran et al. 2002).

Furthermore, DPPC has been demonstrated to inhibit LPS-elicited IL-8 production in A549 lung epithelial cells by blocking TLR4 translocation into lipid raft domains (Abate et al. 2010). Moreover, DPPC has been proved to modulate the production of platelet-

activating factor after LPS stimulation by inhibiting two membrane-associated enzymes involved in its biosynthetic pathway (Tonks et al. 2003); to attenuate LPS-induced respiratory burst via downregulation of protein kinase C (PKC) δ isoform (Tonks et al. 2005); to decrease inflammatory responses independently of mitogen-activated protein kinase (MAPK) activation (Tonks et al. 2001); and to increase cyclooxygenase 2 (COX2) expression and prostaglandin E2 (PGE2) production via phosphorylation of CREB transcription factor, all in a human monocytic cell line (Morris et al. 2008). Interestingly, in these studies the maximum effect was observed after preincubating DPPC with the cells for two hours, and DPPC immunoregulatory properties appeared to be dependent on its uptake by the cells. The authors suggested that DPPC immunoregulatory action could be mediated by a membrane perturbing mechanism.

In another report, internalized DPPC has been shown to regulate the expression of different surface markers in human macrophages, increasing TLR2, TLR4 and scavenger receptor A expression and reducing CD14 membrane levels, among others (Gille et al. 2007). Nevertheless, the mechanism underlying DPPC immunomodulatory properties remains largely unknown.

Anionic phospholipids

Recent studies have revealed that surfactant anionic phospholipids play a critical role as immunosuppressive mediators in the alveolar space, preventing different pathogens and molecules from eliciting an inflammatory immune response. Among these phospholipids, POPG has been the most studied (Numata et al. 2012b).

POPG has been reported to counteract LPS-induced response through different mechanisms. POPG is able to inhibit the binding of LPS to LBP and CD14 (Hashimoto et al. 2003). Furthermore, Kuronuma et al. recently demonstrated that POPG liposomes inhibit TNF-α and nitric oxide productions as well as activation of MAPK and NF-κB pathways induced by LPS in primary rat and human AMs and in U937 cells, by binding to CD14 and MD2 proteins. They also suppress inflammation in LPS-challenged mice (Kuronuma et al. 2009).

POPG also disrupts *Mycoplasma pneumoniae*-induced activation of TLR2 and pro-inflammatory response in RAW 264.7 cells and human macrophages (Kandasamy et al. 2011).

Moreover, POPG is also able to neutralize different respiratory viruses. POPG vesicles provide significant protection in mice infected with influenza A virus. They also attenuate IL-8 release from infected cultured human bronchial epithelial cells. In fact, POPG binds to influenza A virus through a high-affinity interaction and inhibits its attachment to epithelial cells (Numata et al. 2012a). POPG can also suppress respiratory syncytial virus (RSV)-induced inflammation and infection *in vitro* and *in vivo* by directly binding to RSV and blocking viral attachment to epithelial cell surface. It also provides short-term prophylaxis against RSV infection in mice (Numata et al. 2010; Numata et al. 2013a; Numata et al. 2013b).

In these studies, POPG anti-inflammatory action required segregated pools of this phospholipid (Kuronuma et al. 2009; Numata et al. 2010; Kandasamy et al. 2011; Numata et al. 2012a; Numata et al. 2013a; Numata et al. 2013b). Whether POPG is capable of preserving its anti-inflammatory properties in the presence of other surfactant components such as DPPC has not been currently demonstrated.

Apart from POPG, dipalmitoylphosphatidylglycerol (DPPG) (16:0/16:0-PG) also exhibits antiviral activity, since it interacts with Vaccinia virus and blocks its attachment to host cells, preventing infection (Perino et al. 2011). PI has also been found to attenuate LPS-elicited inflammatory response *in vitro* and *in vivo* by binding to CD14 (Kuronuma et al. 2009). Cardiolipin, a minor component of surfactant, strongly inhibits LPS-induced TNF-α release by human mononuclear cells and macrophages (Mueller et al. 2005) and has an immunosuppressive effect during pneumonia after being released from injured cells (Ray et al. 2010).

4.2.2 Surfactant proteins

All surfactant proteins have been demonstrated to be involved to a greater or lesser extent in host immune defense and in the maintenance of lung immunological homeostasis (Chroneos et al. 2010; Glasser and Mallampalli 2012).

Pulmonary collectins

SP-A and SP-D play critical roles in the immune host defense of the lungs and in the maintenance of pulmonary homeostasis (Ariki et al. 2012).

In fact, SP-A and SP-D knockout mice are susceptible to bacterial and viral infections. They exhibit slower clearance of microbes such as *Pseudomonas aeruginosa* (LeVine et al. 1998; Giannoni et al. 2006), *Mycoplasma pulmonis* (Hickman-Davis et al. 1999) or RSV (LeVine et al. 1999; LeVine et al. 2004), among others; and increased inflammation after infection with some pathogens, including influenza type A virus, *Haemophilus influenzae* or group B *Streptococcus* (LeVine et al. 2000; LeVine et al. 2002).

Pulmonary collectins bind to different ligands on the surface of pathogens: LPS on the surface of Gram-negative bacteria, lipoteichoic acid on Gram-positive bacteria, glycoprotein on fungi, lipoarabinomannan on mycobacteria, phospholipids on mycoplasma and surface glycoproteins on viruses. They can therefore agglutinate these microbes, acting as opsonins (Hogenkamp et al. 2007; Ariki et al. 2012). Moreover, they can directly inhibit the growth of some pathogens. For example, SP-A and SP-D have been proved to suppress the growth of Gram-negative bacteria by increasing membrane permeability (Wu et al. 2003). They have also been demonstrated to disrupt *Mycobacterium avium* growth by forming a physical barrier around its surface and restricting nutrient uptake (Ariki et al. 2011). Interestingly, pathogen agglutination and microbial growth inhibition are independent activities (Ariki et al. 2012).

Furthermore, pulmonary collectins are able to regulate the phagocytosis of pathogens by immune cells (Ariki et al. 2012). C1q receptor mediates SP-A-opsonized *Staphylococcus aureus* uptake by monocytes (Geertsma et al. 1994). Moreover, SP-R210 is a specific receptor for SP-A which facilitates the phagocytosis of SP-A-opsonized microbes (Chroneos et al. 1996; Sever-Chroneos et al. 2011). Additionally, pulmonary collectins enhance the expression of scavenger receptor A and mannose receptor in AMs, further improving pathogen internalization (Kudo et al. 2004; Kuronuma et al. 2004). Their interaction with calreticulin and CD91 also increases the clearance of apoptotic cells (Vandivier et al. 2002). Conversely, SP-A and SP-D can also inhibit phagocytosis by AMs through their interaction with signal inhibitory regulatory protein α (SIRPα) (Janssen et al. 2008).

Pulmonary collectins have also been demonstrated to stimulate or attenuate inflammatory responses in a pathogen- and cell-specific manner. For example, SP-A and SP-D have been shown to bind to the extracellular domains of TLR2, TLR4, the TLR4 adaptor protein MD-2 and CD14 through their CRDs. These interactions prevent the recognition of TLR ligands and inhibit TLR-induced signaling and pro-inflammatory cytokine production (Chroneos et al. 2010; Ariki et al. 2012). SP-A and SP-D pro- or anti-inflammatory effect also depends on their binding orientation. When their CRDs are not interacting with microbes, they bind to SIRPα and inhibit inflammation. However, when SP-A interacts with a pathogen through its CRD, its collagenous tail can stimulate an inflammatory response by binding to calreticulin and CD91 (Gardai et al. 2003). Similarly, S-nitrosylated SP-D loses its dodecameric structure and its collagenous domain can also bind to calreticulin and CD91, activating pro-inflammatory signaling (Guo et al. 2008).

Finally, several human diseases have been reported to be associated with polymorphisms in the genes encoding surfactant collectins, including asthma, allergic rhinitis, chronic obstructive pulmonary disease or idiopathic pulmonary fibrosis (Ariki et al. 2012; Silveyra and Floros 2012).

Surfactant protein B

As stated above, SP-B is essential for life, since its absence leads to lethal respiratory failure at birth. This protein is absolutely necessary for the correct synthesis of pulmonary surfactant complexes and also for surfactant proper biophysical functionality (Clark et al. 1995; Melton et al. 2003; Pérez-Gil 2008). Additionally, increasing evidence indicates that SP-B is also involved in the maintenance of pulmonary immune homeostasis (Chroneos et al. 2010; Glasser and Mallampalli 2012).

Studies with inducible transgenic mice have revealed that the reduction of SP-B expression elicits an inflammatory response in AMs and type II AECs, probably caused by surfactant dysfunction, and results in increased alveolar concentrations of the pro-inflammatory cytokines IL-6 and IL-1β and the chemokine macrophage inflammatory protein-2. Restoration of SP-B expression rapidly reverses pulmonary inflammation (Ikegami et al. 2005b).

Additionally, SP-B deficiency has been reported to exacerbate lung injury in LPS-challenged mice (Epaud et al. 2003). Moreover, SP-B has been proved to be able to reduce LPS-induced nitric oxide production by rat AMs (Miles et al. 1999).

Finally, it has also been suggested that SP-B presents significant homologies with some eukaryotic antibiotic peptides (Blanco and Pérez-Gil 2007). Actually, SP-B and SP-B-related peptides have been shown to promote aggregation and killing of bacteria, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and group B *Streptococcus*, by increasing bacterial membrane permeability (Ryan et al. 2006). However, the physiological relevance of SP-B bactericidal activity has still to be further investigated *in vivo*.

Surfactant protein C

As described before, SP-C biophysical function is very important for maintaining the integrity and proper functionality of surfactant multilayered surface film (Pérez-Gil 2008). Nevertheless, SP-C has also been demonstrated to play a very important role in the innate immune host defense of the lungs.

Murine, porcine and rabbit SP-C have been shown to bind to the lipid A region of LPS and to CD14 (Augusto et al. 2001; Augusto et al. 2002; Augusto et al 2003a). The interaction of SP-C with LPS does not require the presence of calcium ions (Augusto et al. 2001) or the palmitoylation of the protein, but is highly dependent on the structure of LPS. Moreover, the positives charges present in the N-terminal segment of SP-C are essential for its binding to LPS (Augusto et al. 2002). Furthermore, SP-C inserted in DPPC vesicles has been proved to inhibit LPS-induced TNF-α production in peritoneal and alveolar macrophages (Augusto et al. 2003b). Additionally, hydrophilic peptides mimicking SP-C N-terminal segment have been reported to bind to LPS to the same extent as porcine SP-C, and to reduce TNF-α synthesis in LPS-stimulated macrophages in the presence of serum, probably by interfering with the formation of LPS/CD14/LBP complexes (Garcia-Verdugo et al. 2009). These studies indicate that SP-C directly neutralizes LPS and prevents it from eliciting a pro-inflammatory response in alveolar cells.

Increasing evidence suggests that SP-C has an essential immunomodulatory function. Mutations in the gene encoding SP-C cause interstitial lung disease in humans, a disease

characterized by structural remodeling of the distal spaces and impaired gas exchange, often leading to the development of fibrosis in children and adults (Glasser et al. 2010; Gower and Nogee 2011). These patients suffer recurrent neonatal and childhood pulmonary infections by respiratory viruses, including influenza virus and RSV (Chibbar et al. 2004; Bullard and Nogee 2007).

These observations have been confirmed with the study of SP-C-deficient mice. These animals have been shown to develop pneumonitis and emphysema (Glasser et al. 2003) and increased and prolonged fibrosis after bleomycin exposure (Lawson et al. 2005). They also present higher susceptibility to *Pseudomonas aeruginosa* infection and AMs with reduced phagocytic activity and altered phenotype (Glasser et al. 2008). In addition to that, SP-C knock-out mice have also been proved to be susceptible to RSV infection, with decreased viral clearance and more robust and sustained pulmonary inflammation (Glasser et al. 2009). Furthermore, genetic replacement of SP-C in these mice reduces RSV-elicited inflammation and restores viral clearance (Glasser et al. 2013a). In LPS-challenged mice, SP-C deficiency leads to increased and prolonged inflammatory injury and enhanced cytokine production by type II AECs in the absence or presence of endotoxin (Glasser et al. 2013b). These studies indicate that the absence of SP-C is linked to dysregulated immune responses and altered activity and phenotype of AMs and type II AECs. However, the mechanism underlying the immunoregulatory functions of SP-C has not been unraveled yet.

5. EXOGENOUS SURFACTANT THERAPY

In 1959, Avery and Mead were the first to suggest that surfactant deficiency was the cause of neonatal RDS (Avery and Mead 1959). In 1973, Enhörning et al. demonstrated it when tracheal instillation of active whole surfactant from adult animals into preterm rabbit pups restored normal lung function (Enhörning et al. 1973a; Enhörning et al. 1973b). Finally, Fujiwara et al. were the first to successfully treat human infants with RDS with an exogenous surfactant (Fujiwara et al. 1980).

Since then, extensive research focusing on pulmonary surfactant biology has helped the development of different exogenous surfactant preparations which differ in origin and composition (Blanco and Pérez-Gil 2007; Willson and Notter 2011).

Surfactant replacement therapy is currently the standard of care for the prevention and treatment of neonatal RDS. It is also routinely used to treat newborns with meconium aspiration syndrome as well as infants with respiratory failure from pneumonia. However, the efficacy and clinical benefits of surfactant therapy in lung injury beyond the neonatal period, such as in adult ALI/ARDS, are not completely demonstrated (Willson and Notter 2011). Moreover, even though surfactant components have been proved to play critical active and immunoregulatory roles in the immune host defense of the lungs, the use of exogenous surfactants as anti-inflammatory or immunomodulatory agents during lung inflammatory diseases has not been studied in clinical trials, let alone approved yet.

5.1 Types of exogenous surfactants

Clinical surfactant preparations can be subcategorized in three different groups (Willson and Notter 2011):

I) Organic solvent extracts of lavaged lung surfactant from animal sources (Bovine lung extract surfactant, Alveofact, Calfactant, among others).
II) Organic solvent extracts of processed animal lung tissue (including surfactant-TA, Curosurf, Survanta).
III) Synthetic preparations without animal-derived material (Lucinactant, Venticute®).

Surfactants in categories I and II are referred to as natural surfactants. They are obtained by organic extraction with organic solvent mixtures of surfactant complexes derived from bronchoalveolar lavages or lung mincing. After that, neutral lipids are usually removed by precipitation with acetone or by chromatography. These surfactant preparations contain all surfactant phospholipids and in general they preserve the presence of SP-B and SP-C hydrophobic peptides. Surfactant obtained by lung mincing can also be contaminated by plasmatic or cellular components. Due to the diversity of sources of the surfactant material (bovine or porcine), of the methods used to process it, and due to the supplementation of natural mixtures with components such as DPPC or palmitic acid (PA), the biochemical composition of natural surfactants varies between one another (Blanco and Pérez-Gil 2007).

Although natural surfactants are considered to have better biophysical and biological activity than synthetic preparations, they also present some drawbacks. There are batch-to-batch variations in composition and potential risk of transmission of microbes. Moreover, their relative high cost makes their use affordable only in developed countries (Zuo et al. 2008).

Synthetic lung surfactant preparations have advantages in purity, reproducibility, manufacturing quality control efficiency and cost. However, designing fully synthetic surfactants with high surface activity equivalent to that of native surfactant has been proved to be a difficult challenge (Willson and Notter 2011).

In the beginning, synthetic surfactant preparations contained only synthetic lipids, but their use was abandoned soon, since their surface activity was lower than that of animal-derived surfactants due to the absence of SP-B and SP-C (Halliday 1995; Mingarro et al. 2008). Thereafter, synthetic surfactants have been supplemented with simplified peptides or recombinant surfactant protein analogs (Zuo et al. 2008; Willson and Notter 2011).

Two synthetic surfactants are currently under clinical study. Lucinactant (Surfaxin®) contains KL4, a 21-residue peptide inspired on the structure of SP-B. It consists of leucine aminoacids with a lysine moiety introduced every five residues. KL4 enhances the adsorption of DPPC/POPG/PA mixtures, and physiological concentrations of SP-A improve this adsorption (Sáenz et al. 2006; Sáenz et al. 2007). KL4-containing surfactant has been shown to be very effective in clinical trials of human RDS (Cochrane et al. 1996; Sinha et al. 2005). And interestingly, airway lavage with diluted KL4-containing surfactant improves lung function in experimental and clinical meconium aspiration syndrome (Cochrane et al. 1998) and in ARDS patients (Wiswell et al. 1999).

The other one (Venticute®) is a synthetic pulmonary surfactant constituted of synthetic lipids and recombinant human SP-C expressed in bacteria (Mingarro et al. 2008; Willson and Notter 2011).

5.2 The synthetic pulmonary surfactant based on recombinant human SP-C

This synthetic surfactant preparation (sSPC) is composed of DPPC, POPG, PA, human recombinant SP-C and $CaCl_2$ (63.4%, 27.8%, 4.5%, 1.8% and 2.5% by weight, respectively). Recombinant SP-C is expressed in bacteria and its sequence is based on the

sequence of the human protein. However, the cysteine residues at positions 4 and 5 have been replaced by phenylalanines and the methionine at position 32 with isoleucine. The rationale behind the cysteine to phenylalanine replacement is that palmitoylation cannot be effectuated in bacteria. Free cysteine residues could form intra- or inter-molecular disulfide bonds (Spragg et al. 2000; Mingarro et al. 2008). In addition to that, the SP-C sequence of some animals has a phenylalanine instead of one of the palmitoylated cysteines (Foot et al. 2007). Phenylalanine residues have high propensity to partition into the membrane interface (White and Wimley 1998), and could mimic at least in part the role of a palmitoylated cysteine (Mingarro et al. 2008). On the other hand, the replacement of the methionine with isoleucine avoids uncontrolled oxidation (Spragg et al. 2000).

In spite of SP-C modifications, this synthetic surfactant has been reported to have a very good surface activity. Moreover, it has been proved to be effective treating preterm rabbits and lambs (Davis et al. 1998) and in animal models of lung injury (Häfner et al. 1998; Lewis et al. 1999; Spragg et al. 2000; Ikegami and Jobe 2002).

One of the objectives of synthetic surfactant development is to find a surfactant formulation able to resist inactivation and to treat adult ALI/ARDS, a complex pathology in which inflammation as a secondary consequence of several types of direct or indirect lung injury promotes the disruption of the alveolar-capillary barrier and leads to the leakage of plasma components into the airspaces. These molecules are responsible for the biophysical inactivation of lung surfactant (Mingarro et al. 2008; Willson and Notter 2011).

The efficacy of the synthetic surfactant based on recombinant human SP-C treating ARDS patients has been studied in several clinical trials. In a phase II clinical trial, ARDS patients treated with sSPC tended to have improved gas exchange and survival and reduced need for mechanical ventilation (Spragg et al. 2003). In another phase II clinical trial conducted in parallel, sSPC treatment resulted in better gas exchange, normalization of alveolar phospholipid and protein contents, and improvement but not normalization of alveolar surfactant surface activity (Markart et al. 2007). In a phase III study, patients treated with this synthetic surfactant had increased blood oxygenation, but similar mortality or need for mechanical ventilation than patients receiving standard therapy (Spragg et al. 2004). However, *post hoc* analysis revealed that sSPC treatment was associated with reduced mortality in patients suffering respiratory insufficiency due to pneumonia or

aspiration (Taut et al. 2008). These patients were the focus of another clinical trial. In this last study, patients treated with the synthetic surfactant based on recombinant human SP-C did not present clinical benefit, probably due to a new surfactant resuspension step that could have partially inactivated the surfactant preparation (Spragg et al. 2011). Therefore, the efficacy of sSPC in adult ALI/ARDS has not entirely been demonstrated yet.

Interestingly, some studies have reported sSPC anti-inflammatory and immunoregulatory actions. In one of the previously mentioned phase II clinical trials, the synthetic surfactant based on recombinant human SP-C decreased IL-6 levels in bronchoalveolar lavages of patients suffering ARDS (Spragg et al. 2003). In addition to that, sSPC has been shown to modulate the viability of human eosinophils *in vitro* (Erpenbeck et al. 2009). Moreover, sSPC was also proved to reduce TNF-α expression and to enhance IL-10 production after stimulation with LPS in a human monocytic cell line (Wemhöner et al. 2009). However, the role of each of sSPC components and their mechanism of action have not been analyzed yet.

These later studies and the extensive evidence existing about the critical active and immunoregulatory roles played by the different pulmonary surfactant components in lung host defense raise this synthetic surfactant based on recombinant human SP-C as a good tool to study the regulatory functions of each of its components in the alveolar innate immune system.

OBJECTIVES

The lungs are continuously exposed to a diverse array of inhaled particles, allergens and pathogens that escape the defense mechanisms of the upper respiratory airways. To remain healthy, the lungs must have an immune response able to kill and clear pathogens without triggering an excessive inflammatory response, which would damage alveolar tissues, compromising lung homeostasis and gas exchange.

In the alveolar space, alveolar macrophages (AMs) and alveolar epithelial cells (AECs) are continuously in contact with pulmonary surfactant, a complex network of extracellular membranes synthesized and secreted into the alveolar space by type II AECs. Surfactant consists of approximately 90% of lipids (mainly phospholipids) and contains four associated proteins [surfactant proteins A, B, C and D (SP-A, SP-B, SP-C and SP-D)] (Casals and Cañadas 2012). The main function of surfactant is to reduce the surface tension at the air-liquid interface in order to prevent the lungs from collapsing at the end of expiration. Surfactant deficiency in immature lungs is the main cause of neonatal respiratory distress syndrome (RDS) (Whitsett and Weaver 2002).

In addition to its biophysical relevance, surfactant has also been recognized to play an essential role in the immune host defense of the lungs (Wright 2005; Chroneos et al. 2010; Ariki et al. 2012; Glasser and Mallampalli 2012). Although the mechanism underlying the immunoregulatory action of surfactant components is being elucidated, some points are still unknown. Furthermore, in the alveolar space surfactant is endocytosed by AMs and type II pneumocytes in order to assure its degradation and recycling (Casals and Cañadas 2012; Agassandian and Mallampalli 2013). The effect of these internalized surfactant components on the phenotype or the immune response of AMs and AECs remains largely unknown.

Synthetic surfactants are currently being developed to treat RDS. Among them, a synthetic pulmonary surfactant based on recombinant human SP-C (sSPC) is composed of 98% of lipids by weight [dipalmitoylphosphatidylcholine (DPPC)/ palmitoyloleoylphosphatidylglycerol (POPG)/palmitic acid (PA) 2.3:1:0.16 w/w] and 2% of recombinant human SP-C. sSPC surfactant has been shown to be effective in animal models of lung injury (Lewis et al. 1999; Spragg et al. 2000; Ikegami and Jobe 2002) and treating acute respiratory distress syndrome (ARDS) patients (Spragg et al. 2003; Spragg et al. 2004; Markart et al. 2007). Furthermore, Spragg et al. (2003) observed that the synthetic surfactant based on recombinant human SP-C presented a potential anti-inflammatory

action, since the treatment with sSPC decreased the levels of interleukin (IL)-6 in bronchoalveolar lavages of patients suffering ARDS (Spragg et al. 2003). In addition to that, sSPC synthetic surfactant was also shown to reduce tumor necrosis factor α (TNF-α) expression in a human monocytic cell line stimulated with bacterial lipopolysaccharide (LPS) (Wemhöner et al. 2009). A greater knowledge about the potential modulatory action of the different components present in the synthetic surfactant based on recombinant human SP-C on the immune response of alveolar cells to viruses or endotoxins is important in order to understand their possible beneficial effect on the treatment of respiratory diseases.

Therefore, the **main objective of this thesis** was to study the immunoregulatory properties of the synthetic surfactant based on recombinant human SP-C and to unravel new details about the mechanism of action of each of its components. In order to accomplish this task, we used two stimuli that have been reported to induce a strong pro-inflammatory response in AMs and AECs:

- Bacterial LPS, which is a component of the outer membrane of Gram-negative bacteria that can promote the development of acute lung injury and ARDS as a consequence of pulmonary bacterial infections or sepsis (Matute-Bello et al. 2008).
- Respiratory syncytial virus (RSV), which is a common and highly contagious respiratory virus that infects airway epithelial cells and other structural and resident immune cells, inducing a strong pro-inflammatory response which promotes virus clearance but can also be detrimental to the host (Lotz and Peebles 2012).

This thesis is composed of three chapters with the following concrete objectives:

1) To determine the extracellular anti-inflammatory effect of the synthetic surfactant based on recombinant human SP-C on the inflammatory response of LPS-stimulated mouse alveolar and peritoneal macrophages (chapter 1).

2) To evaluate the intracellular immunomodulatory action of synthetic surfactant vesicles internalized by alveolar macrophages on the activation state of these cells after being stimulated with LPS (chapter 2).

3) To investigate the intracellular immunoregulatory functions of internalized synthetic surfactant vesicles on the immune response of human alveolar epithelial cells (A549 cells) infected with human respiratory syncytial virus (chapter 3).

MATERIALS AND METHODS

1. CELL CULTURE

Two murine macrophage cell lines were used in this thesis: MH-S alveolar macrophages (figure 1) (Mbawuike and Herscowitz 1989) and RAW 264.7 peritoneal macrophages (figure 2) (Raschke et al. 1978). These cell lines were both purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD, USA) at 37ºC under a humidified 5% CO_2 atmosphere.

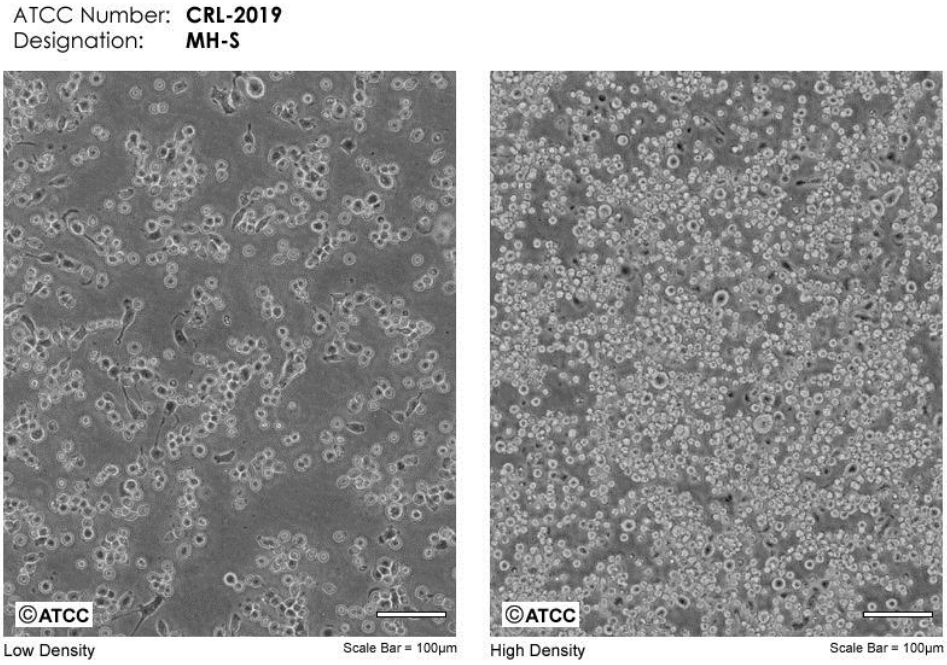


Figure 1: Optic micrograph showing MH-S morphology.

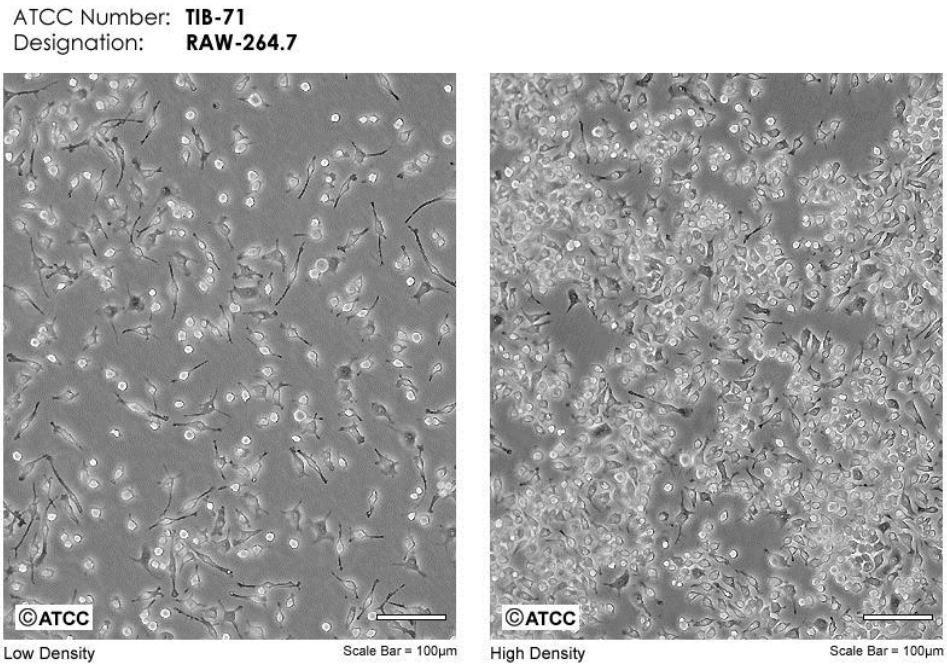


Figure 2: Optic micrograph showing RAW 264.7 morphology.

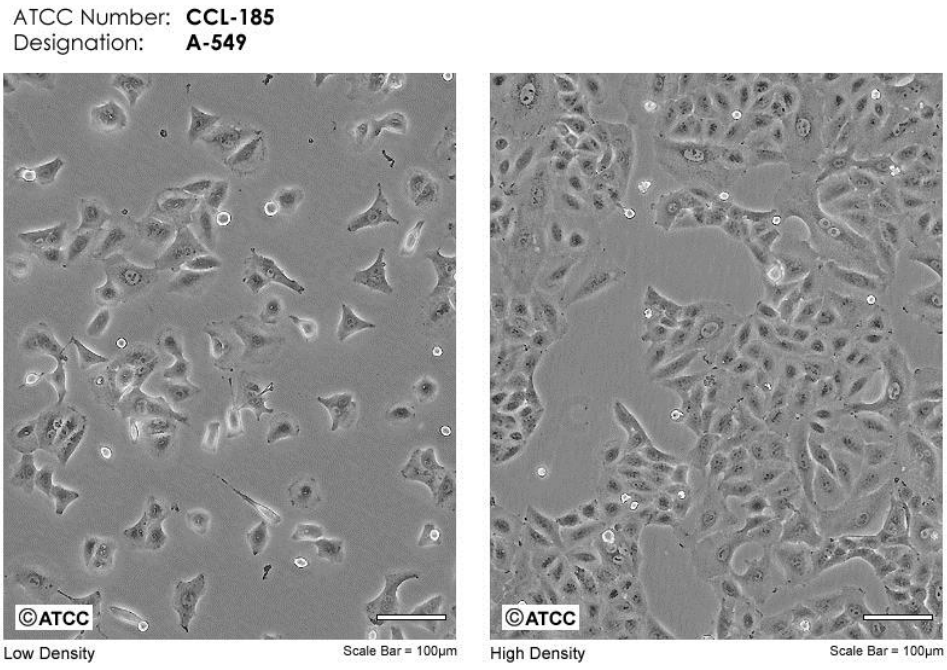


Figure 3: Optic micrograph showing A549 morphology.

Two human cell lines were used in the experiments realized with respiratory syncytial virus: A549 alveolar epithelial cells (figure 3) (Giard et al. 1973), and HEp-2 epithelial cells (figure 4) (Moore et al. 1955). These cell lines were both purchased from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (Lonza Group Ltd, Basel, Switzerland) and 10% fetal calf serum (FCS) (Linus, Madrid, Spain) at 37ºC under a humidified 5% CO_2 atmosphere.

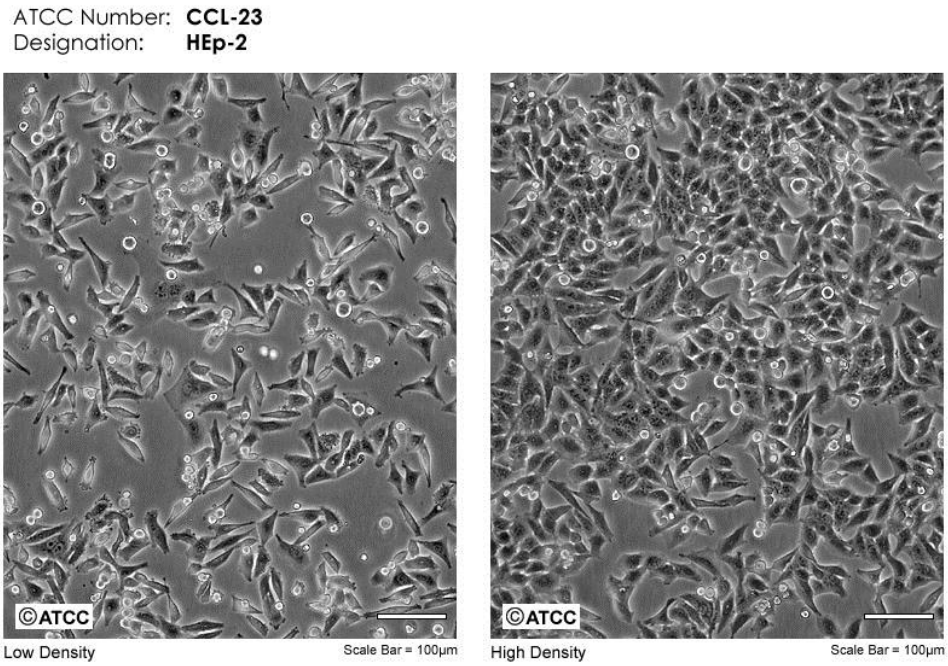


Figure 4: Optic micrograph showing HEp-2 morphology.

2. LIPID PREPARATIONS

2.1 Preparation of synthetic surfactant vesicles

The synthetic surfactant based on recombinant human surfactant protein C (SP-C) (sSPC) as well as the analogue without SP-C (sPL) were provided by Nycomed GmbH (Konstanz, Germany, now Takeda Pharmaceutical Company Limited, Osaka, Japan). This synthetic surfactant powder contains dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylglycerol (POPG), palmitic acid (PA), human recombinant SP-

C and $CaCl_2$ (63.4%, 27.8%, 4.5%, 1.8% and 2.5% by weight, respectively). Synthetic surfactant dried mixture was hydrated in 0.9% (w/v) sterile-filtered NaCl, allowing it to swell for one hour at a temperature above the gel-to-liquid phase transition temperature (T_m, see section 3.3) (45ºC) to obtain multilamellar vesicles (MLVs). These were sonicated at the same temperature for 8 minutes at 390 W/cm^2 (bursts of 0.6 s and 0.4 s between bursts) in a UP200S sonifier with a 2 mm microtip (Hielscher Ultrasonics, Teltow, Germany) in order to enrich the solution in small unilamellar vesicles (SUVs).

2.2 Preparation of phospholipid vesicles

DPPC, POPG and PA (Avanti Polar Lipids, Alabaster, AL, USA) were dissolved in chloroform/methanol (2:1 v/v) at 100 mg/ml. DPPC, POPG, DPPC/PA (2.3:0.16 w/w), DPPC/POPG (2.3:1 w/w) and DPPC/POPG/PA (2.3:1:0.16 w/w) mixtures were evaporated to dryness under a gentle nitrogen stream and traces of solvent were subsequently removed by evacuation under reduced pressure overnight. Dry residues were hydrated in 20 mM Tris, 150 mM NaCl buffer at a final concentration of 10 mg/ml, allowing them to swell for one hour at a temperature above the gel-to-liquid phase transition temperature (50ºC for DPPC vesicles, room temperature for POPG liposomes, and 40ºC for the mixtures) to obtain MLVs. SUVs were obtained as described in section 2.1.

3. LIPID VESICLE CHARACTERIZATION

3.1 Dynamic Light Scattering (DLS)

DLS, sometimes referred to as Quasi-Elastic Light Scattering, is a technique that allows to measure the size distribution of molecules and particles dispersed or dissolved in a liquid. The Brownian motion of the scattering particles causes fluctuations in light scattering intensity that are measured as a function of time. The velocity of the Brownian motion of the particles can be calculated and related to their hydrodynamic size (Philo 2006).

sSPC and sPL MLVs and SUVs were prepared as described in section 2.1, at a final concentration of 250 μg/ml in 0.9% NaCl. SUVs were incubated at 37ºC for 0, 2, 4 or 24 hours. The size of the vesicles was then measured at 25°C in a Zetasizer Nano S (Malvern Instruments, Malvern, UK) equipped with a 633-nm HeNe laser. Three scans were

performed for each sample. The hydrodynamic diameter was calculated using the general purpose algorithm available from the Malvern software for DLS analysis, which correlates the diffusion coefficient to the hydrodynamic diameter through the Stokes-Einstein equation, eq. 1:

$$d_H = \frac{k_B\, T}{3\pi\, \eta D} \tag{1}$$

where d_H is the hydrodynamic diameter, k_B is the Boltzmann constant, T is the temperature, η is the viscosity, and D is the translational diffusion coefficient. The multiple narrow modes algorithm was also used to verify the results obtained by the general purpose method.

3.2 Zeta potential measurement

In solution, a charged particle is surrounded by counter-ions over a region called electric double layer, which can be divided into an inner region of strongly bound ions called the stern layer, and a region of loosely bound ions called the diffuse layer (figure 5A). Some of these ions move with the particle in the solution. The limit from which the ions do not follow the movement of the particle is called the slipping plane, which is located in some point of the diffuse layer. Zeta potential is the potential existing in this slipping plane.

sSPC and sPL SUVs were prepared at a final concentration of 1 mg/ml in 5 mM Tris buffer (pH 7.2) as described in section 2.1. DPPC, POPG, DPPC/PA (2.3:0.16 w/w), DPPC/POPG (2.3:1 w/w) and DPPC/POPG/PA (2.3:1:0.16 w/w) SUVs were prepared as explained in section 2.2 at the same final concentration in 5 mM Tris buffer (pH 7.2) containing 0.22 mM $CaCl_2$. Zeta potential measurement was performed with a Zetasizer Nano S (Malvern Instruments), applying an electric field across the dispersion. This measurement is based on the fact that charged particles within the dispersion migrate toward the electrode of opposite charge. The velocity of this movement is proportional to the magnitude of the zeta potential and is determined by laser Doppler anemometry (figure 5B). The frequency shift of an incident laser beam provides the particle electrophoretic mobility, which is converted to the zeta potential with the application of the Henry equation, eq 2:

$$U_E = \frac{2\varepsilon Z f(\kappa a)}{3\eta} \quad (2)$$

where U_E is the electrophoretic mobility, ε is the dielectric constant, Z is the zeta potential, η is the viscosity and $f(\kappa a)$ is Henry's function. The parameter a refers to the radius of the particle, and κ is the Debye parameter (Henry 1931; Doane et al. 2012). Three scans were recorded for each sample.

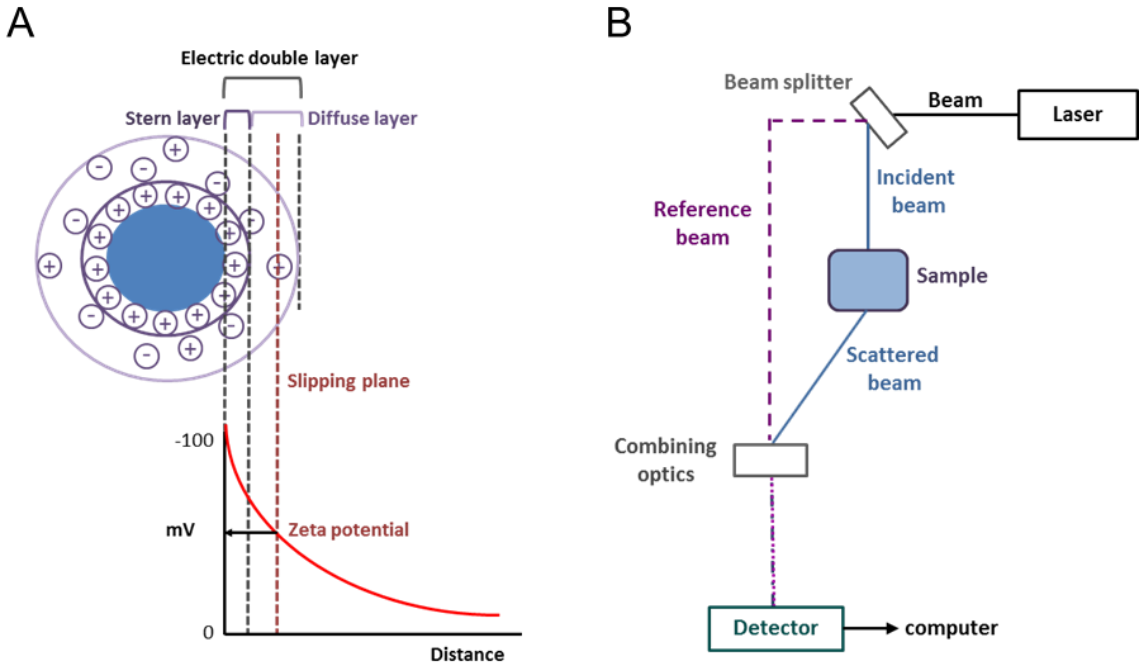


Figure 5: (A) Schematic representation of a particle with negative surface charge and its zeta potential. (B) Schematic representation of the optical configuration of the Zetasizer Nano for zeta potential measurement. The laser beam is split into a reference beam and an incident beam directed through the sample. An electric field is applied in the sample cell. The recombination of the two beams provides the electrophoretic mobility of the particles in the solution.

3.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a technique which measures the thermodynamic properties of temperature-induced phase transitions. A sample and an appropriate reference material (which does not undergo a phase transition at the scanned temperatures, usually the same buffer in which the sample has been prepared) are

simultaneously heated in different cells at the same predetermined rate. If the sample undergoes a thermally induced phase transition, absorption or release of heat takes place (depending on whether the process is endothermic or exothermic). The instrumental control system has to supply more (or less) heat to the sample cell to maintain its temperature equal to that of the reference cell.

The recorded parameter in a DSC scan is the excess heat capacity (C_p) of a sample with respect to the reference as a function of temperature. Endothermic transitions are represented as positive peaks. Three thermodynamic parameters can be directly determined from the thermogram (figure 6). Firstly, the phase transition temperature, T_m, corresponds to the maximum C_p of the peak. For symmetric curves, T_m represents the temperature at which the transition is half complete. Secondly, the relative cooperativity of the process is related to the sharpness of the peak, which is commonly expressed as the temperature width at half-height of the peak, $\Delta T_{1/2.}$ And finally, the enthalpy of the transition (ΔH) can be calculated by integrating the area below the thermogram (McElhaney 1986; Cañadas and Casals 2013).

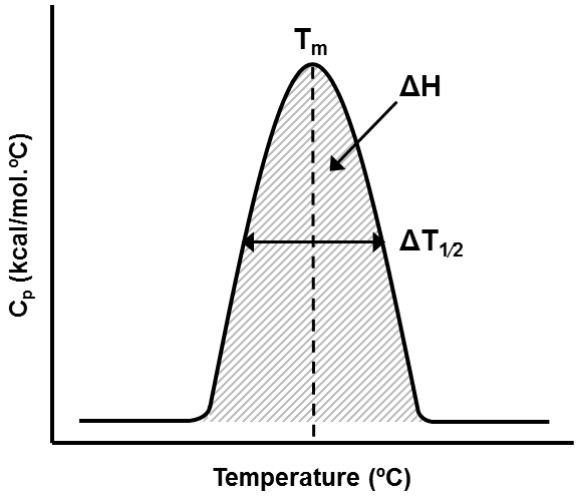


Figure 6: DSC thermogram for a two-state endothermic process. T_m is the phase transition temperature, $\Delta T_{1/2}$ is the temperature width at half-height of the peak, and ΔH is the enthalpy of the thermodynamic event.

As it has been explained in the introduction, lipid bilayers and biological membranes undergo a temperature-induced phase transition from a relatively ordered crystalline-like state (gel phase (L_β)) to a relatively disordered fluid-like state (liquid-crystalline phase (L_α)). The properties of the gel-to-liquid phase transition of different lipid mixtures can be therefore studied by DSC.

sSPC and sPL MLVs were prepared at a final concentration of 1 mg/ml in 150 mM NaCl, 5 mM Tris buffer, pH 7.2 (buffer A). DPPC, DPPC/PA (2.3:0.16 w/w), DPPC/POPG (2.3:1 w/w) and DPPC/POPG/PA (2.3:1:0.16 w/w) MLVs were prepared at the same final concentration in buffer A containing 0.22 mM $CaCl_2$. Calorimetric measurements were performed in a Microcal VP differential scanning calorimeter (Microcal Inc., Northampton, MA, USA) at a heating rate of 0.5°C per minute from 10ºC to 80ºC. Vesicles were loaded in the sample cell of the microcalorimeter with 0.6 ml of buffer A (with or without 0.22 mM $CaCl_2$, depending on the sample) in the reference cell. Three calorimetric scans were recorded for each sample. The Origin software (OriginLab Corporation, Northampton, MA, USA) was used for data acquisition and analysis. The excess heat capacity functions were obtained after subtraction of the buffer-buffer base line.

3.4 Giant Unilamellar Vesicle (GUV) preparation and observation

GUVs composed of sPL, sSPC, DPPC/POPG (2.3:1 w/w) and DPPC/POPG/PA (2.3:1:0.16 w/w) were prepared from lipid samples dissolved in chloroform by using the electroformation method originally developed by Angelova and Dimitrov (Angelova and Dimitrov 1986) using indium tin oxide (ITO) electrodes. Samples were labeled with 1% (w/w) rhodamine-phosphatidylethanolamine (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA).

Films were formed by spreading small drops of the lipid solutions on the surface of aluminosilicate glass slides coated with ITO. Another ITO slide was attached to the other one with VITREX. Then the chamber was placed under low vacuum for 30 minutes to allow the material to adsorb onto the ITO slides. Once the material was adsorbed, 1 ml of an aqueous solution (200 mM sucrose), previously heated to the desired temperature (above the lipid mixture T_m), was added to the chamber. The electrodes were immediately connected to a function generator, and a low frequency alternating current field (sinusoidal wave function with a frequency of 10 Hz and a peak-to-peak voltage of 2.1 V) was applied for 4 hours at 60ºC, followed by some minutes at 4 Hz to detach the GUVs from the ITO slide. Vesicles were then collected with a pipette and transferred to a plastic tube.

Aliquots of GUVs suspended in sucrose were added to an isoosmolar concentration of glucose solution (208 mM). The density difference between the interior and exterior of the

GUVs induced the vesicles to sink to the bottom of the chamber, and within a few minutes the vesicles were ready to be observed. GUV preparations were observed with a Nikon Eclipse TG 2000-U fluorescence microscope (Nikon Corporation, Tokyo, Japan) in a slide covered with a cover slip separated with a plastic ring.

3.5 Smooth lipopolysaccharide (LPS) interaction with surfactant vesicles

sPL or sSPC MLVs at 250 μg/ml were mixed with 1 μg/ml smooth LPS from *Escherichia coli* (055:B5) (Sigma, St. Louis, MO, USA) in 0.9% sterile-filtered NaCl and incubated for 30 minutes with soft swelling at 37ºC in pyrogen-free glass tubes (Cambrex, East Rutherford, NJ, USA). Mixtures were centrifuged for 15 minutes at 15000 x g. Supernatant aliquots were diluted 1:20000 in order to determine LPS concentration using Limulus Amebocyte Lysate (LAL) QCL-1000 LPS detection kit (Cambrex), following the manufacturer's instructions.

Briefly, samples were mixed with LAL (lyophilized lysate prepared from the circulating amebocytes of the horseshoe crab *Limulus polyphemus*) and incubated at 37ºC for 10 minutes. In this step, LPS catalyzed the activation of a proenzyme present in the LAL. Then, the colorless substrate Ac-Ile-Glu-Ala-Arg-p-nitroaniline was added to the mixtures and incubated at 37ºC for 6 minutes. The activated enzyme catalyzed the splitting of p-nitroaniline from the substrate, giving a yellow color to the solution. The reaction was stopped with 10% (w/v) sodium dodecyl-sulphate (SDS) and the absorbance was read at 405 nm in a DU.800 spectrophotometer (Beckman Coulter, Brea, CA, USA). LPS concentration in the samples was calculated from a standard curve.

4. EXPERIMENTS WITH MH-S AND RAW 264.7 MACROPHAGES

Dose and time course experiments were initially conducted in order to determine the optimal concentration and time for cell stimulation depending on the analyzed molecules.

4.1 Cell viability analysis

Cell viability was controlled before and after each experiment by two different methods.

4.1.1 Propidium iodide exclusion

Cells were harvested, washed with phosphate buffered saline (PBS), incubated for 5 minutes with 50 μg/ml propidium iodide (Sigma) and analyzed with a FACSCalibur cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Propidium iodide is excluded from viable cells, but in dead cells it binds to deoxyribonucleic acid (DNA) by intercalating between the bases, increasing its fluorescence by 20- to 30-fold (Arndt-Jovin and Jovin 1989). Therefore, the percentage of non-viable cells was determined as the percentage of cells that presented increased fluorescence intensity.

4.1.2 Trypan-blue exclusion

An aliquot of cells was mixed with 0.4% (w/v) trypan-blue in PBS, loaded in a hemacytometer, and observed with a TMS Nikon microscope. As trypan-blue does not penetrate viable cells, the percentage of dead cells was determined as the percentage of cells that showed a blue color.

4.2 Phenotypic analysis

MH-S or RAW 264.7 macrophages were seeded at $1x10^6$ cells per well in 6-well plates (TPP, Trasadingen, Switzerland) in 2 ml of 10% FBS RPMI medium and were grown overnight at 37ºC. Then, cells were stimulated with smooth LPS from *E. coli* (055:B5) (Sigma) (1 μg/ml) for 24 hours. Cells were harvested and washed with 1 mM ethylenediaminetetraacetic acid (EDTA) in PBS. To prevent nonspecific binding, macrophages were incubated with 10% FBS medium for 15 minutes on ice, before the addition of a rat anti-mouse primary antibody against CD14 (eBioscience, San Diego, CA, USA), CD206 (AbD Serotec, Kidlington, UK) and CD16 (Stemcell Technologies, Vancouver, Canada); or an isotype-specific control (AbD Serotec) at a final concentration of 10 μg/ml for 30 minutes. After that, cells were washed with PBS and stained with a fluorescein isothiocyanate (FITC)-conjugated anti-rat secondary antibody (AbD Serotec) diluted in 10% FBS medium for 30 minutes in the dark. Phenotypic analysis was performed with a FACSCalibur cytometer (BD Biosciences).

4.3 Intracellular reactive oxygen species (ROS) detection

MH-S or RAW 264.7 macrophages were plated at $2.5x10^5$ cells per well in 24-well plates (TPP) in 1 ml of 2% FBS RPMI medium without phenol red (Gibco, Invitrogen) and were grown overnight at 37ºC. Then cells were stimulated with 1 μg/ml smooth LPS from *E. coli* (055:B5) (Sigma) for 24 hours. The membrane permeable dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma) was added at a final concentration of 5 μM for 30 minutes. Cells were harvested and the formation of the fluorescent-oxidized derivative of the dye was detected by flow cytometry with a FACSCalibur cytometer (BD Biosciences).

4.4 Measurement of cytokine release

MH-S or RAW 264.7 macrophages were seeded at $2.5x10^5$ cells per well in 24-well plates (TPP) in 1 ml of 5% FBS RPMI medium and grown overnight at 37ºC. Cells were then stimulated with 1 or 2 μg/ml smooth LPS from *E. coli* (055:B5) (Sigma). In the experiments with surfactant or lipid vesicles, these were added immediately before LPS addition, or for a preincubation time that depends on the experiments (as indicated in every figure legend). After that, supernatants were collected, cleared by centrifugation for 5 minutes at 10000 x g and stored at -80ºC. Tumor necrosis factor α (TNF-α) and interleukin (IL)-10 concentrations were determined using commercially available enzyme-linked immune sorbent assay (ELISA) kits (BD Biosciences) following the manufacturer's instructions.

Briefly, a Maxisorp® polystyrene plate (Nunc, Rochester, NY, USA) was coated overnight at 4ºC with a primary antibody against TNF-α or IL-10. Between every step, plates were washed with 0.05% tween-20 in PBS. Wells were blocked with 10% FBS in PBS to prevent nonspecific binding for one hour, and incubated with the samples or standards for two hours. After that, plates were incubated with a biotinylated detection antibody against TNF-α or IL-10 and streptavidin-horseradish peroxidase conjugate for another hour. Tetramethylbenzidine (TMB) (BD Biosciences) was used as substrate. The reaction was stopped with 2 M H_2SO_4 and absorbance was read at 450 nm in a microplate reader (Asys Hitech GmbH, Eugendorf, Austria).

4.5 Quantitative real-time polymerase chain reaction (qPCR)

MH-S cells were plated at $1x10^6$ cells per well in 6-well plates (TPP) in 2 ml of 5% FBS RPMI medium. Macrophages were then stimulated with 1 μg/ml smooth LPS from *E. coli* (055:B5) (Sigma) for one hour. In the experiments with surfactant vesicles, these were added immediately before LPS addition, or for a preincubation time that depends on the experiments (as indicated in every figure legend). Culture medium was then discarded and cells were scrapped, transferred to a 15 ml tube and washed with cold PBS. Cell pellets were frozen in liquid N_2 and stored at -80ºC.

4.5.1 Ribonucleic acid (RNA) extraction

RNA extraction was performed using High Pure RNA Isolation kit (Roche Diagnostics, Basel, Switzerland), following the manufacturer's instructions. Briefly, cell pellets were lysed in 400 μl of lysis/binding buffer and 200 μl of PBS and homogenized with a syringe coupled to a 25-gauge needle. Samples were transferred to a high pure filter tube coupled to a collection tube, in which nucleic acids are retained in two layers of glass fiber fleece. Residual contaminating DNA was digested incubating the samples with 1.8 U/μl deoxyribonuclease I (DNase I) for 30 minutes at room temperature. Bound RNA was washed several times with different buffers supplied in the kit to eliminate salts, proteins, cellular impurities and inhibitory contaminants. RNA was finally recovered in 90 μl of elution buffer, quantified in a Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and stored at -80ºC.

4.5.2 RNA reverse transcription

Complementary DNA (cDNA) was synthesized using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). The reaction was carried out in 20 μl of final volume containing 1 μg of total RNA, 60 μM random hexamer primers, 1 U/μl Protector ribonuclease (RNase) Inhibitor, 1 mM Deoxynucleotide mix, 0.5 U/μl Transcriptor reverse transcriptase, Transcriptor RT reaction buffer and RNase-free water. Firstly, RNA was mixed with the random hexamer primers in 13 μl of volume and incubated for 10 minutes at 65ºC in a Thermal-cycler C-1000 (Bio-Rad Laboratories, Berkeley, CA, USA) to assure RNA denaturation. Then, the rest of reagents were added and the mixtures were incubated

for 20 minutes at 25ºC (to allow primer annealing), one hour at 55ºC (for cDNA synthesis) and 5 minutes at 85ºC (to inactivate the enzyme). cDNA was stored at -20ºC.

4.5.3 qPCRs performed using the Universal Probe Library (UPL) (Roche Diagnostics) system

Briefly, qPCR reactions were carried out in 10 µl of final volume using 0.2 µM primers, 100 nM probe, 2 µl of 1:10 diluted cDNA (without diluting in the case of IL-4 qPCRs), FastStart Universal Probe Master kit [ROX] (Roche Diagnostics) and nuclease-free water. Reactions were performed in an ABI PRISM 7900 HT-Fast (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). The amplification program was: 10 minutes at 95ºC, followed by 40 cycles of denaturation for 15 seconds at 95ºC and primer annealing and extension for 1 minute at 60ºC.

Probes were selected using ProbeFinder Software (Roche Diagnostics) and purchased from Roche Diagnostics. Primers were designed using Lasergene Software (DNAStar, Madison, WI, USA) and Blastn (NCBI) for specific amplification of the sequence of interest, and manufactured in Sigma (table 1). Assays were performed in triplicates. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), and results were normalized to the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1: Primers and probes used in qPCRs performed using the UPL system.

Gene	Forward primer	Reverse primer	UPL probe
TNF-α	5'-CGGTGCCTATGTCTCAGCCTC-3'	5'-GGCCATTTGGGAACTTCTCATC-3'	49
iNOS	5'-GCACAAAGGGCTCAAAGGAG-3'	5'-CTCTCTTGCGGACATCTC-3'	101
IL-4	5'-CATCGGCATTTTGAACGAG-3'	5'-GCAGCTCACTCTCTGTGGTG-3'	2
GAPDH	5'-CTGGCATGGCCTTCCGTG-3'	5'-CCTGCTTCACCACCTTCTTG-3'	80

4.5.4 qPCRs performed using predesigned primers

Primers and probes (carboxyfluorescein (FAM) dye-labeled and minor groove binder (MGB)-quenched) for the genes indicated in table 2 were products from Assays-on-demand (TaqMan quantitative gene expression assays, Applied Biosystems).

Table 2: Gene expression assays used in qPCRs performed using predesigned primers.

Gene symbol	Gene name	Gene expression assay
Arg 1	arginase 1	Mm00475988_m1
Car3	carbonic anhydrase 3	Mm01281795_m1
Car5a	carbonic anhydrase 5a	Mm00500371_m1
Cd200r3	cluster of differentiation 200 receptor 3	Mm01343888_m1
Cd80	cluster of differentiation 80	Mm00711659_m1
Chi3l3	chitinase 3-like 3	Mm00657889_mH
Cxcl10	C-X-C motif chemokine 10	Mm01345163_g1
Cxcl11	C-X-C motif chemokine 11	Mm01345188_g1
Enpp2	autotaxin	Mm00516572_m1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
Ifna4	interferon alpha 4	Mm00833969_s1
Infb1	interferon beta 1	Mm00439552_s1
Infg	interferon gamma	Mm01168134_m1
Irf1	interferon regulatory factor 1	Mm01288580_m1
Ptgs2	cyclooxygenase 2	Mm00478474_m1
Socs3	suppressor of cytokine signaling 3	Mm00545913_s1

Reactions were carried out in 10 µl of final volume using 0.5 µl of gene expression assay, 2 µl of 1:10 diluted cDNA, TaqMan Gene Expression master mix (Applied Biosystems) and nuclease-free water. Reactions were performed in an ABI PRISM 7900 HT-Fast (Applied Biosystems). The amplification program was: 10 minutes at 95ºC, followed by 40 cycles of denaturation for 15 seconds at 95ºC and primer annealing and extension for 1 minute at 60ºC. Assays were performed in triplicates. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), and results were normalized to the expression levels of GAPDH.

4.6 Western-blotting

MH-S macrophages were seeded as explained in section 4.4. In the experiments with surfactant or lipid vesicles, these were added immediately before LPS addition, or for a preincubation time that depends on the experiments (as indicated in every figure legend).

Cells were then stimulated with 1 μg/ml smooth LPS from *E. coli* (055:B5) (Sigma) for 10 minutes (to detect extracellular signal-regulated kinase (ERK), p·38 and Akt phosphorylations), 20 minutes (to detect glycogen synthase kinase 3 (GSK3) α/β phosphorylation), 30 minutes (to analyze IκB-α phosphorylation), or 6 hours (to determine inducible nitric oxide synthase (iNOS) protein levels). Supernatants were removed and cell plates were frozen in liquid N_2.

4.6.1 Analysis of ERK, p38, Akt, and IκB-α phosphorylations

Cells were lysed on ice in 250 μl of lysis buffer (10 mM Hepes, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM Na_3VO_4, 10 mM NaF, 10 mM sodium pyrophosphate and 20 mM β-glycerophosphate). Insoluble cell debris was removed by centrifugation at 10000 x g for 10 minutes at 4ºC. Protein content was measured using Bradford reagent (Bio-Rad) (Bradford 1976) (see section 4.7.1). Lysate aliquots with 10 μg of total protein were separated by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions using 10% acrylamide gels, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Blots were blocked with methanol followed by air-drying and probed overnight with the appropriate primary phospho-specific antibodies against phospho-ERK (p44/42) (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser473) and phospho-IκB-α (Ser32/36) (Cell Signaling, Beverly, MA, USA) diluted in 0.05% tween-20 Tris buffered saline (TBS). Membranes were washed with 0.05% tween-20 TBS and probed with a secondary horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling) for one hour. After washing the membranes again, blots were developed using enhanced chemiluminescent (ECL) substrate (Millipore, Billerica, MA, USA).

To detect total proteins or GAPDH (as loading controls) membranes were incubated for 30 minutes at 50ºC with stripping buffer (0.5 M Tris, pH 6.8, containing 0.7% (v/v) β-mercaptoethanol and 2% (w/v) SDS). Then, membranes were probed as explained before using primary antibodies against ERK (p44/42), p38, Akt or GAPDH (Cell Signaling).

Band intensities were calculated with QuantityOne software (Bio-Rad).

4.6.2 Detection of iNOS protein levels

Cells were lysed as described above using the same lysis buffer without Na_3VO_4, NaF, sodium pyrophosphate and β-glycerophosphate. Protein content was measured using Bradford reagent (Bio-Rad) (Bradford 1976) (see section 4.7.1). Lysate aliquots with 6 μg of total protein were separated by SDS-PAGE in reducing conditions using 8% acrylamide gels, and transferred to a PVDF membrane (Bio-Rad). Blots were blocked with TBS containing 5% (w/v) nonfat dry milk and 0.05% (v/v) tween-20 for one hour. Then, membranes were washed with 0.05% tween-20 TBS and probed overnight with primary antibodies against iNOS or GAPDH (Cell Signaling) diluted in TBS containing 2.5% (w/v) nonfat dry milk and 0.05% (v/v) tween-20. Membranes were washed and probed with a secondary HRP-conjugated antibody (Cell Signaling) for one hour. After washing the membranes again, blots were developed using ECL substrate (Millipore).

Band intensities were calculated with QuantityOne software (Bio-Rad).

4.6.3 Analysis of GSK3α/β phosphorylation

Cells were lysed on ice in 250 μl of a different lysis buffer (25 mM Tris, pH 7.4, containing 25 mM NaCl, 1 mM EDTA, 0.2% (v/v) Triton X-100, 0.1% (w/v) SDS, 1 mM PMSF, 1 mM benzamidine, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM Na_3VO4, 10 mM NaF, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate and 25 nM okadaic acid). Insoluble cell debris was removed by centrifugation at 10 000 x g for 10 minutes at 4ºC, and supernatants were homogenized using a syringe coupled to a 25-gauge needle. Protein content was measured using Bicinchoninic Acid Kit for Protein Determination (Sigma) following the supplier's instructions (see section 4.7.2). Lysate aliquots with 6 μg of total protein were separated by SDS-PAGE in reducing conditions using 10% acrylamide gels, and transferred to a PVDF membrane (Bio-Rad). Blots were blocked with TBS containing 2.5% (w/v) nonfat dry milk and 0.05% (v/v) tween-20 for one hour. Then, membranes were washed with 0.05% tween-20 TBS and probed overnight with a primary antibody against phospho-GSK3α/β (Ser21/9) (Cell Signaling) diluted in TBS containing 1.25% (w/v) nonfat dry milk and 0.05% (v/v) tween-20. Membranes were washed and probed with a secondary HRP-conjugated antibody (Cell Signaling) for one hour. After washing the membranes again, blots were developed using ECL substrate (Millipore).

To detect total GSK3α/β or GAPDH (as loading controls) membranes were stripped as explained in section 4.6.1, and probed as detailed above with primary antibodies against GSK3α/β or GAPDH (Cell Signaling).

Band intensities were calculated with QuantityOne software (Bio-Rad).

4.7 Determination of total protein content

Two different methods were used to obtain the total protein concentration of cell lysates.

4.7.1 Bradford assay

This method involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976). Under acidic conditions, this dye is predominantly in a doubly protonated red cationic form. In the presence of proteins, the dye binds to basic (especially arginine) and aromatic amino acid residues (Compton and Jones 1985) and is converted to a stable unprotonated blue form. Briefly, 5 μl of sample were mixed with 250 μl of Bradford reagent (Bio-Rad) in a polystyrene 96-well plate (Nunc) and were incubated for 5 minutes at room temperature. The absorbance was read at 595 nm in a microplate reader (Asys Hitech). The protein concentration in the samples was calculated from a bovine serum albumin standard curve.

4.7.2 Bicinchoninic acid (BCA) assay

This assay is based on the fact that cysteine, tryptophan, tyrosine, and peptide bonds are able to reduce Cu^{2+} ions to Cu^{1+} ions (Wiechelman et al. 1988). The amount of reduction is proportional to the protein concentration. In basic conditions, BCA forms a stable purple-blue complex with Cu^{1+} ions, providing a method to quantify protein content (Smith et al. 1985). To carry out this assay, 10 μl of sample were mixed with 80 μl of the working reagent supplied in the Bicinchoninic Acid Kit for Protein Determination (Sigma) in 96-well polystyrene plates (Nunc). The working reagent was prepared mixing 50 parts of BCA alkaline solution (reagent A) with 1 part of 4% (w/v) copper (II) sulfate pentahydrate solution (reagent B). The plate was incubated for 30 minutes at 37ºC, and absorbance was

read at 570 nm in a microplate reader (Asys Hitech). The protein concentration in the samples was calculated from a bovine serum albumin standard curve.

4.8 Immunofluorescence microscopy

Macrophages were plated as described in section 4.4. Previously, a round cover slide was U.V. irradiated on each well for sterilization. sSPC and sPL vesicles were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, also known as $DiIC_{18}$ (DiI) (Invitrogen), in a 200:1 phopholipids:probe molar ratio, by hydrating lyophilized lipids and the appropriate volume of probe diluted in dimethyl sulfoxide (DMSO) in 0.9% sterile-filtered NaCl and allowing them to swell for one hour at 45ºC. Then, lipids were sonicated as explained in section 2.1 and added to the cells at a final concentration of 100 or 250 μg/ml for different incubation times (as indicated in the figure legends). Cells were washed with PBS and fixed in 3.8% paraformaldehyde for 30 minutes. Then, macrophages were blocked with 10% FBS in PBS, and stained with a rat primary antibody against mouse CD14 (eBioscience) diluted in 10% FBS in PBS for one hour. Cells were then washed thrice with PBS and incubated for 30 minutes with a secondary anti-rat FITC-conjugated antibody (AbD Serotec) diluted in 10% FBS in PBS. Finally, cover slides were washed and mounted on slides with Mowiol containing 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for nuclei staining. Micrographs were taken on a Leica TCS SP2 Confocal system (Leica Camera AG, Solms, Germany).

In endocytosis inhibition assays, the following inhibitors were added 30 minutes before the addition of DiI-labeled vesicles: 2.5 mM amantadine, 100 μM monodansylcadaverine (both clathrin-dependent endocytosis inhibitors), 50 μg/ml nystatin (a caveolae-dependent endocytosis inhibitor) (Ivanov 2008), 25 μM monensin (a ionophore that prevents endosomal maturation) (Mollenhauer et al. 1990) and 0.1 μM bafilomycin A1 (specific inhibitor of the vacuolar proton pump) (Gagliardi et al. 1999).

4.9 Surfactant vesicle endocytosis quantification

MH-S alveolar macrophages were seeded as described in section 4.4. Synthetic surfactant DiI-labeled vesicles were prepared as explained above. They were added to the cells at a final concentration of 250 μg/ml for 24 hours. Then, cells were harvested, washed

with PBS, and vesicle endocytosis was quantified using a FACSCalibur cytometer (BD Biosciences).

4.10 Inhibition of GSK3α/β with lithium chloride

4.10.1 Measurement of cytokine release after GSK3α/β inhibition

MH-S macrophages were plated as explained in section 4.4, and preincubated with 250 μg/ml sPL SUVs for 18 hours. Then, 10 mM LiCl was added to the cells for one hour, prior to the stimulation with LPS (1 or 2 μg/ml) for 4 hours. After that, TNF-α and IL-10 secretions were determined by ELISA as detailed in section 4.4.

4.10.2 Quantification of the expression of inflammatory markers after GSK3α/β inhibition

MH-S cells were seeded as explained in section 4.5, and preincubated with 250 μg/ml sPL SUVs for 18 hours. Then, 10 mM LiCl was added to the cells for one hour, prior to the stimulation with LPS (1 μg/ml) for another hour. After that, RNA was extracted, cDNA was synthesized and the expressions of IRF1, COX2, CXCL10, CXCL11 and CD80 were determined by qPCR as explained in section 4.5.

5. EXPERIMENTS WITH RESPIRATORY SYNCYTIAL VIRUS

5.1 Virus propagation and purification

The Long strain of human respiratory syncytial virus (RSV) was propagated in HEp-2 cells in 2% FCS DMEM medium. Viruses were purified from clarified culture supernatants by polyethylene glycol precipitation and centrifugation in a 30–45% discontinuous sucrose gradient in TNE buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) (Mbiguino and Menezes 1991).

Briefly, HEp-2 cells were seeded at $5x10^6$ cells per P100 plate (TPP) in 10% FCS DMEM medium, grown overnight at 37ºC, and infected at a multiplicity of infection (moi) of 0.5 plaque-forming units (pfu) per cell in 2% FCS DMEM medium. At 48 hours post-infection, cells were scrapped and homogenized 3 times with a syringe coupled to a 20-gauge needle.

Then, 10 more P150 plates with HEp-2 cells were infected with the virus obtained from the first plate diluted in 2% FCS DMEM medium. At 48 hours post-infection, supernatants were collected and cells were scrapped. Both were mixed and homogenized 3 times with a syringe coupled to a 20-gauge needle. The mix was then clarified by centrifugation at 4000 x g for 20 minutes at 4ºC. After that, 50% polyethylene glycol 6000 was added to the supernatant to a final concentration of 10%, and the mix was maintained for 90 minutes at 4ºC with soft swelling. After that, it was centrifuged at 4000 x g for 20 minutes at 4ºC.

Pellets were suspended in TNE buffer, homogenized 5 times with a syringe coupled to a 20-gauge needle and sonicated 3 times for 10 seconds. Samples were then added to a 30–45% discontinuous sucrose gradient in TNE buffer and centrifuged at 150000 x g for 90 minutes at 4ºC. The white band corresponding to the virus, placed between the 30% and 45% sucrose solutions, was finally recovered. Virus samples were then aliquoted and stored in liquid N_2. Virus titers were thereafter determined by plaque assay in layered HEp-2 cells (see next section).

5.2 Virus titration

A549 cells were plated at $3.6x10^5$ cells per well in 6-well plates (TPP) in 2 ml of 10% FCS DMEM medium, grown overnight at 37ºC, and incubated with 250 μg/ml sSPC SUVs for 18 hours. Then they were mock-infected (with sucrose) or infected with purified human RSV at a moi of 3 pfu per cell in 2% FCS DMEM medium. At 16, 24, 32, or 48 hours post-infection, supernatants were collected, cleared by centrifugation and stored in liquid N_2.

HEp-2 cells were seeded at $1.2x10^6$ cells per well in 6-well plates (TPP) in 2 ml of 10% FCS DMEM medium, and grown overnight at 37ºC. A549 culture supernatants were diluted in 2% FCS DMEM medium, and 200 μl were added to the HEp-2 monolayers. Cells were maintained at 37ºC for 90 minutes to allow virus adsorption. After that, 3 ml of 0.5% low melting-point agarose (Conda, Madrid, Spain) in 2% FCS DMEM medium were added to each well. Plates were incubated at 4ºC for 30 minutes to solidify the agarose, and then at 37ºC for 5 days. Cells were then fixed with 4% formaldehyde in PBS, agarose was removed, and cells were fixed again with methanol, washed twice with PBS and incubated with 1% serum albumin in PBS for 30 minutes at room temperature. After another washing

step, cells were incubated for an hour with a mixture of monoclonal antibodies against the two major glycoproteins of the virus (2F, 47F, 56F, 021/1G, 021/2G) (García-Barreno et al. 1989; Martínez et al. 1997) diluted 1:50 in 1% serum albumin PBS. Cells were washed again and incubated for one hour with an anti-mouse IgG HRP-linked whole antibody (Amersham Biosciences, GE Healthcare, Little Chalfont, Buckinghamshire, UK) diluted 1:500 in 1% serum albumin PBS. Finally, plaques were visualized using 3-amino-9-ethylcarbazole (Sigma). Virus titers were calculated using the following equation, eq 3:

$$T = \frac{ND}{V} \tag{3}$$

where *T* is the virus titer expressed in pfu/ml, *N* is the number of plaques obtained, *D* is the dilution factor of the tested culture supernatant, and *V* is the volume of the aliquot of culture supernatant expressed in ml.

5.3 Quantitative real-time polymerase chain reaction (qPCR)

A549 cells were plated at $3.6x10^5$ cells per well in 6-well plates (TPP) in 2 ml of 10% FCS DMEM medium, grown overnight at 37ºC, and incubated with 100 or 250 μg/ml sSPC or sPL SUVs for 18 hours. Then they were mock-infected (with sucrose) or infected with purified human RSV at a moi of 3 pfu per cell in 2% FCS DMEM medium. After 90 minutes of adsorption, fresh medium was added and cells were maintained at 37ºC for 24 hours. Then they were harvested, washed with PBS, frozen in liquid N_2 and stored at -80ºC.

5.3.1 RNA extraction

RNA extraction was performed using RNeasy Mini kit (Qiagen, Hilden, Germany) following the supplier's instructions. Briefly, cells were disrupted in 600 μl of lysis buffer (buffer RLT) and homogenized with a syringe coupled to a 20-gauge needle. The same volume of 70% ethanol was then added to the samples, and these were transferred to a RNeasy spin column placed in a collection tube. Total RNA was bound to the spin column membrane by centrifugation and washed several times with different buffers supplied in the kit to eliminate salts, proteins, cellular impurities and inhibitory contaminants. Residual contaminating DNA was digested incubating the samples with 337.5 U/μl DNase I (RNase-free DNase Set (Qiagen)) for 15 minutes at room temperature. RNA was finally recovered

in 100 µl of RNase-free water, quantified with a spectrophotometer Biophotometer Plus (Eppendorf, Hamburg, Germany) and stored at -80ºC.

5.3.2 RNA reverse transcription

cDNA was synthesized with the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer's instructions. Briefly, the reaction was carried out in 100 µl of final volume containing 2.5 µg of total RNA, random primers, deoxynucleotide triphosphate (dNTP) mix, 2.5 U/µl MultiScribe^{TM} reverse transcriptase, reverse transcription buffer and nuclease-free water. Reagents were mixed and samples were incubated for 10 minutes at 25ºC (to allow primer annealing), for 2 hours at 37ºC (to synthesize the cDNA) and for 5 minutes at 85ºC (to inactivate the enzyme) in a thermomixer (Eppendorf). Finally, cDNA was stored at -20ºC.

5.3.3 qPCR procedure

qPCRs were performed using TaqMan quantitative gene expression assays (Applied Biosystems). Primers and probes (FAM dye-labeled and MGB-quenched) for the genes indicated in table 3 were products from Assays-on-demand (Applied Biosystems).

A Custom TaqMan Gene Expression Assay (Applied Biosystems) was used for amplification and quantification of the nucleoprotein RNA of human RSV (forward primer: 5´CATGATTCTCCTGATTGTGGGATGA3´, reverse primer: 5´TCACGGCTGTAAGACCAGATCTAT3´, probe: 5´CCCCTGCTGCCAATTT3´).

Table 3: Primers and probes used in qPCRs.

Gene symbol	Gene name	Gene expression assay
ACTB	Beta actin	Hs99999903_m1
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Hs00204833_m1
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	Hs01911452_s1
ISG15	ISG15 ubiquitin-like modifier	Hs01921425_s1
TLR3	Toll-like receptor 3	Hs01551078_m1
TNFAIP3	Tumor necrosis factor alpha-induced protein 3	Hs00234713_m1

Reactions were carried out in 30 μl of final volume using 1.5 μl of gene expression assay, 1.8 μl of cDNA (45 ng of cDNA-converted RNA approximately), TaqMan Universal PCR master mix no AmpErase UNG (Applied Biosystems) and nuclease-free water. Assays were performed in triplicate on a StepOne Real Time PCR system (Applied Biosystems). The amplification program was: 3 minutes at 94ºC, followed by 35 cycles of denaturation for 30 seconds at 94ºC, primer annealing for 45 seconds at 65ºC and extension for 45 seconds at 72ºC. After that, samples were incubated for 2 minutes at 72ºC, and finally maintained at 4ºC. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), and results were normalized to the expression levels of β-actin.

5.4 Western-blotting

A549 cells were plated and infected as described in section 5.3. After 90 minutes of virus adsorption, fresh medium was added and cells were maintained at 37ºC for 2.5 or 5 hours to analyze Akt, ERK, p38 and IκB-α phosphorylations, or for 24 hours to detect SP-C levels. Supernatants were removed and cell plates were frozen in liquid N_2.

Cells were lysed on ice in 150 μl of lysis buffer (10 mM Hepes, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM PMSF, 1 mM benzamidine, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM Na_3VO_4, 10 mM NaF, 10 mM sodium pyrophosphate and 20 mM β-glycerophosphate). Protein content was measured using Bicinchoninic Acid Kit for Protein Determination (Sigma) following the manufacturer's instructions (see section 4.7.2).

As insoluble cell debris was not removed by centrifugation in order to prevent the loss of transmembrane proteins, nucleic acids might have interfered in the separation by SDS-PAGE. To avoid this, 100 mM $MgCl_2$ was added to the samples before boiling them and loading them in the polyacrylamide gel.

5.4.1 Analysis of ERK, p38, Akt and IκB-α phosphorylations

Protein separation by SDS-PAGE, gel transference and immunoblots were performed as described in section 4.6.1.

5.4.2 Detection of SP-C levels

Lysate aliquots with 10 μg of total protein were separated by SDS-PAGE using 16% acrylamide gels, and transferred to a PVDF membrane (Bio-Rad). The vacuum-driven system SNAP (Millipore) was used to reduce the incubation times during the western-blot. Between incubations, membranes were washed with PBS containing 0.05% (v/v) tween-20. Blots were blocked with PBS containing 0.1% (w/v) nonfat dry milk and 0.05% (v/v) tween-20, and probed firstly with primary rabbit antibodies against human SP-C (Seven Hills Bioreagents, Cincinnati, OH, USA) or GAPDH (Cell Signaling) (diluted respectively 1:1500 and 1:2000 in 0.05% tween-20 PBS), and secondly with a secondary HRP-conjugated antibody (Sigma) (diluted 1:1500 in 0.05% tween-20 PBS). Blots were developed using ECL substrate (Millipore), and band intensities were calculated with QuantityOne software (Bio-Rad).

6. STATISTICAL ANALYSIS

Quantitative data were analyzed with Sigma-plot software (Systat Software Inc., Chicago, IL, USA). Data are expressed as means ± standard deviations (SD) for biophysical experiments and as means ± standard errors (SE) for cellular experiments. A One-way analysis of variance (ANOVA) followed by a Bonferroni test was performed to compare three or more groups. A Student's t-test was used to compare two groups between them. Differences between means were considered statistically significant when the confidence level was higher than 95% ($p<0.05$).

CHAPTER 1

Phase segregation in surfactant vesicles facilitates phosphatidylglycerol inhibitory actions on the inflammatory response induced by bacterial lipopolysaccharide

1. ABSTRACT

Bacterial lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria that induces a strong pro-inflammatory response in alveolar macrophages (AMs) and can promote the development of acute lung injury and acute respiratory distress syndrome. Among pulmonary surfactant components, dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylglycerol (POPG) and surfactant protein C (SP-C) have been shown to counteract LPS stimulation, but the mechanism of their effect needs to be further studied. The aim of this work was to analyze the extracellular anti-inflammatory effect of a synthetic surfactant, composed of DPPC, POPG, palmitic acid (PA) and recombinant human SP-C on the inflammatory response of mouse alveolar and peritoneal macrophages stimulated with LPS. We found that synthetic surfactant vesicles inhibited LPS-elicited activation of signaling pathways and production of tumor necrosis factor α (TNF-α) and inducible nitric oxide synthase (iNOS). Surfactant vesicles carried out their anti-inflammatory action in the extracellular medium, because their effect decreased as they were endocytosed by the cells. SP-C attenuated only iNOS mRNA production and protein levels, indicating that surfactant lipid component was responsible for the disruption of LPS-induced response. More precisely, we determined that vesicles without POPG were unable to inhibit TNF-α secretion after LPS stimulation. Importantly, we observed by fluorescence microscopy of giant unilamellar vesicles that the presence of PA in DPPC/POPG/PA vesicles enhanced their anti-inflammatory action by increasing ordered/disordered phase segregation and enrichment of POPG in disordered domains. These data provide evidence that POPG is responsible for the inhibition of LPS-induced inflammatory response by synthetic surfactant vesicles, and that ordered/disordered phase segregation is essential for improving synthetic surfactant immunoregulatory properties.

2. INTRODUCTION

Bacterial LPS is a component of the outer membrane of Gram-negative bacteria ubiquitously present in the environment (Augusto et al. 2003b). When LPS reaches the alveolar space as a consequence of an infection or inhalation, it stimulates AMs, which are the main cells of the innate immune system in the lungs (Martin and Frevert 2005). Firstly, LPS molecules bind to LPS-binding protein (LBP) present in serum. LBP transfers LPS to

the cluster of differentiation 14 (CD14), which can be found in a soluble form or anchored to AM cell membrane (Wright et al. 1990; Hailman et al. 1994). Then, CD14 presents LPS to its receptor complex in the surface of AMs, composed of Toll-like receptor 4 (TLR4) and MD-2 (Poltorak et al. 1998; Nagai et al. 2002). The interaction of LPS with the TLR4/MD2 complex triggers the oligomerization of TLR4 and subsequent activation of phosphatidylinositol-3-kinase (PI3K) (O'Toole and Peppelenbosch 2007), nuclear factor-κB (NF-κB) transcription factor and extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs). This leads to production of pro-inflammatory mediators such as TNF-α and iNOS (reviewed in Bode et al. 2012). As a consequence of pulmonary bacterial infections or sepsis, LPS can promote the development of acute lung injury and acute respiratory distress syndrome (ARDS), which are clinical syndromes characterized by lung inflammation, fluid accumulation in the alveoli, impaired blood oxygenation and high mortality and morbidity rates (Matute-Bello et al. 2008).

In the alveolar space, LPS also encounters pulmonary surfactant, a complex network of extracellular membranes containing four associated proteins (surfactant proteins A, B, C, and D (SP-A, SP-B, SP-C and SP-D)). The main function of surfactant is to reduce the surface tension at the air-liquid interface in order to prevent the lungs from collapsing at the end of expiration. Surfactant deficiency in immature lungs is the main cause of neonatal respiratory distress syndrome (RDS) (Whitsett and Weaver 2002).

Pulmonary surfactant components have been shown to contribute to host immune defense and to counteract LPS-induced inflammation. SP-A and SP-D, also known as pulmonary collectins, have bactericidal activity, act as opsonins and modulate AM phagocytic activity and immune response (Ariki et al. 2012). They have also been demonstrated to interact with LPS of various phenotypes (Lim et al. 1994; Kalina et al. 1995). SP-C and SP-B hydrophobic proteins also play an important role in the regulation of the immune response in the lungs. SP-B deficiency causes lung dysfunction and inflammation in mice (Ikegami et al. 2005b) and exacerbates lung injury in LPS-challenged mice (Epaud et al. 2003). SP-C deficient mice are more susceptible to pulmonary infections and injury associated with dysregulated immune responses (Glasser et al. 2003; Lawson et al. 2005; Glasser et al. 2008; Glasser et al. 2009; Glasser et al. 2013a). Moreover, SP-C has

been shown to bind to LPS and CD14 (Augusto et al 2003a), and SP-C inserted in DPPC vesicles inhibits LPS-induced TNF-α production in peritoneal and alveolar macrophages (Augusto et al. 2003b). Phospholipids have also been proved to have immunomodulatory properties. DPPC attenuates LPS-responses in lung epithelial cells (Abate et al. 2010), and in human monocytes (Tonks et al. 2003; Tonks et al. 2005). Furthermore, segregated pools of POPG have been shown to suppress respiratory syncytial virus and influenza A virus infections (Numata et al. 2010; Numata et al. 2012a), to disrupt TLR2 ligand-induced pro-inflammatory responses (Kandasamy et al. 2011) and to inhibit LPS-induced inflammation (Kuronuma et al. 2009). Although the mechanism underlying these immunoregulatory actions is being elucidated, some points are still unknown. Lipid and protein components coexist in native surfactant monolayers, so further studies must be carried out to determine if SP-C is able to bind to LPS in more complex lipid mixtures, or if there is some mechanism by which POPG could preserve its anti-inflammatory action in the presence of other surfactant components such as DPPC, which is the most abundant phospholipid molecular species in surfactant monolayers (Bernhard et al. 2001).

Synthetic surfactants are currently being developed to treat RDS. Among them, a synthetic pulmonary surfactant based on recombinant human SP-C has been shown to be effective in an animal model of lung injury (Ikegami and Jobe 2002) and treating ARDS patients (Spragg et al. 2003; Spragg et al. 2004). Furthermore, it has been shown to modulate LPS-elicited cytokine mRNA expression in a human monocytic cell line (Wemhöner et al. 2009). This synthetic surfactant is composed of DPPC, POPG, PA and recombinant human SP-C. All these characteristics raise this synthetic surfactant as a good tool to study the regulatory functions of each of its components in LPS-induced inflammation in the alveolar space.

The purpose of this study was to evaluate the extracellular anti-inflammatory effect of the synthetic surfactant based on recombinant human SP-C on the inflammatory response of LPS-stimulated mouse alveolar and peritoneal macrophages. More specifically, the aims were to find out which of its components exerted this effect and by which mechanism.

3. EXPERIMENTAL DESIGN

The synthetic surfactant based on recombinant human SP-C contains 98% of lipids by weight (DPPC/POPG/PA 2.3:1:0.16 w/w) and 2% of human recombinant SP-C. SP-C is a small hydrophobic peptide with a α-helix segment which adopts a transmembrane orientation, and a more polar N-terminal segment with two palmitoylated cysteines (Vandenbussche et al. 1992a). However, recombinant SP-C is not palmitoylated. Its sequence is based on the sequence of the human protein, but the cysteine residues at positions 4 and 5 have been replaced by phenylalanines and the methionine at position 32 with isoleucine (Spragg et al. 2000). Surfactant small unilamellar or multilamellar vesicles (SUVs or MLVs) were prepared as described in materials and methods, in a solution containing 150 mM NaCl and 2.2 mM $CaCl_2$. Surfactant vesicles were characterized by dynamic light scattering (DLS), differential scanning calorimetry (DSC), zeta potential measurement and observation of giant unilamellar vesicles (GUVs) by fluorescence microscopy (figure 1).

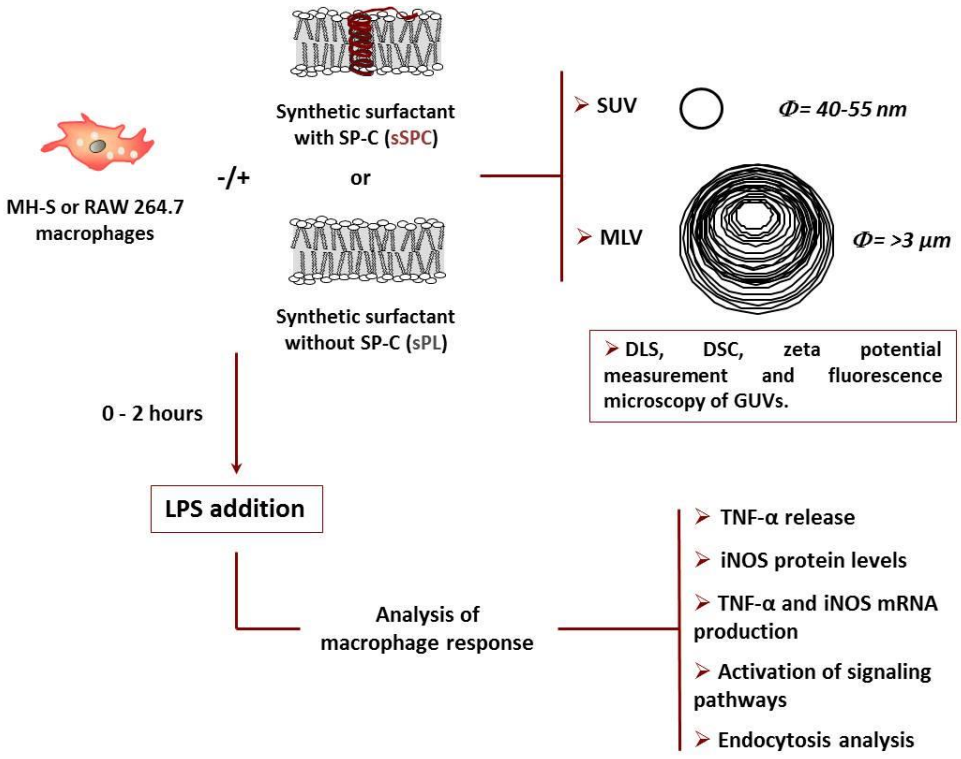


Figure 1: Experimental design used in chapter 1.

To assess the anti-inflammatory action of surfactant vesicles upon LPS-stimulation, we have used two murine macrophage cell lines: MH-S AMs and RAW 264.7 peritoneal macrophages. The cells were stimulated with smooth bacterial LPS from *E. coli* in the presence or absence of synthetic surfactant membranes containing human recombinant SP-C (sSPC), or surfactant membranes without SP-C (sPL) (figure 1).

In some experiments, macrophages were preincubated with sSPC or sPL vesicles for different times not longer than two hours before LPS addition. In other assays, macrophages were stimulated with LPS in the presence or absence of vesicles with different lipid composition, as DPPC, POPG, DPPC/PA (2.3:0.16 w/w), DPPC/POPG (2.3:1 w/w) and DPPC/POPG/PA (2.3:1:0.16 w/w), instead of synthetic surfactant vesicles.

LPS-induced TNF-α release was measured by enzyme-linked immune sorbent assay (ELISA), TNF-α and iNOS mRNA productions were quantified by quantitative real-time polymerase chain reaction (qPCR), iNOS protein levels and activation of signaling pathways were analyzed by western-blot and vesicle endocytosis was studied by confocal microscopy.

4. RESULTS

4.1 Comparison of MH-S and RAW 264.7 response to LPS

In this chapter, two murine macrophage cell lines have been used: MH-S AMs and RAW 264.7 peritoneal macrophages. Initial experiments were conducted in order to compare LPS-induced response in these cells. First of all, we performed a phenotypic analysis to study changes in the expression of three surface markers after LPS stimulation: CD14, which binds to LPS and presents it to TLR4/MD2 receptor complex; mannose receptor, CD206, which is considered a typical marker of M2 alternatively activated macrophages; and FcγRIII receptor for immunoglobulin G, CD16, which is commonly expressed in M1 classically activated macrophages (Mantovani et al. 2004). As we can see in figure 2A, CD14 expression was augmented in RAW 264.7 cells (1.8 ± 0.2 fold increase) but not in MH-S AMs after 24 hours of stimulation with LPS. On the other hand, figures 2B and 2C show that CD206 and CD16 expressions were significantly increased after the stimulation with LPS only in MH-S cells, but to a lesser extent than in RAW 264.7 peritoneal macrophages.

We next examined LPS-elicited production of intracellular reactive oxygen species (ROS). After 24 hours of stimulation with LPS, the production of ROS increased to a higher extent in RAW 264.7 macrophages (2.1 ± 0.2 fold increase) than in MH-S cells (1.16 ± 0.05 fold increase) (figure 3).

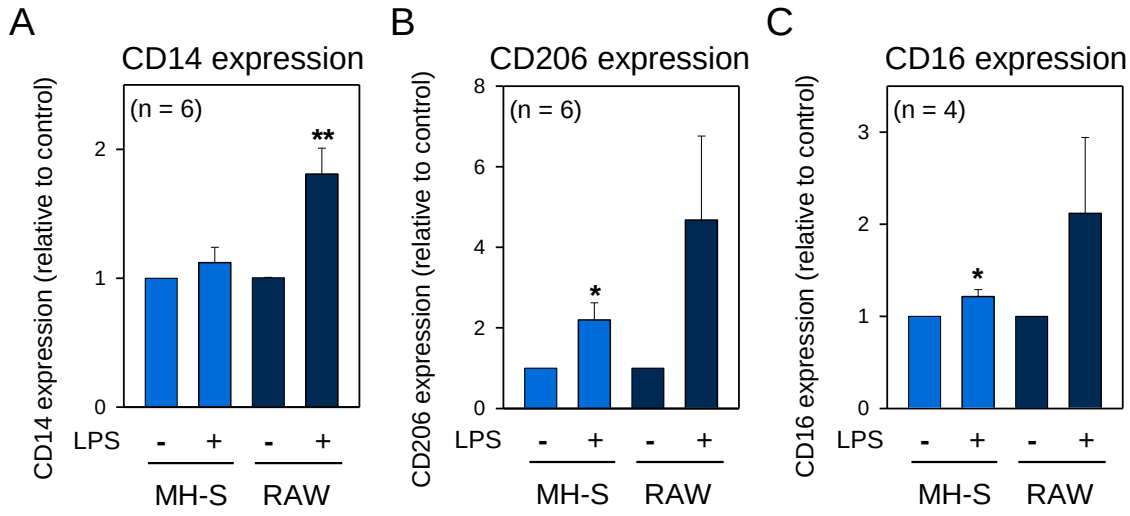


Figure 2: Phenotypic analysis of RAW 264.7 and MH-S cells after being stimulated with LPS (1 μg/ml) for 24 hours. Macrophage surface markers (CD14 (A), CD206 (B) and CD16 (C)) were labeled with FITC-conjugated antibodies and their expression was analyzed by flow cytometry. Data are expressed in arbitrary mean fluorescence intensity units relative to control cells ± standard error (SE). (*p<0.05, **p<0.01 vs control).

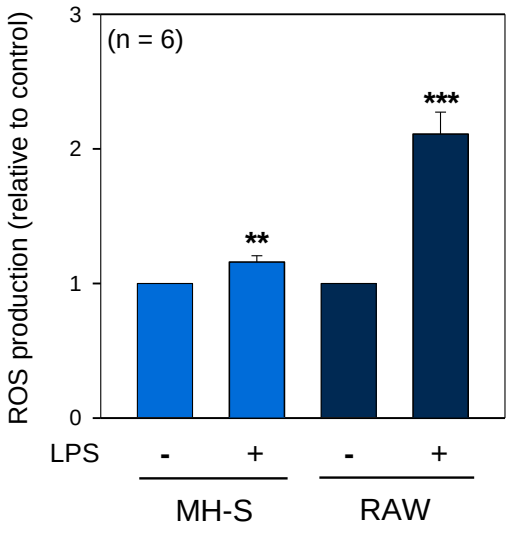


Figure 3: LPS-stimulation induced an increase in ROS production in RAW 264.7 cells to a higher extent than in MH-S cells. Macrophages were stimulated with LPS (1 μg/ml) for 24 hours. Then, the membrane permeable dye DCFH-DA was added at a final concentration of 5 μM for 30 minutes. The formation of the fluorescent-oxidized derivative of the dye was detected by flow cytometry. Data are expressed as arbitrary mean fluorescence intensity units relative to control ± SE. (**p<0.01, ***p<0.001 vs control).

We also conducted time course experiments to compare LPS-induced TNF-α and interleukin- (IL)-10 secretions (figure 4). MH-S AMs released less TNF-α (pro-inflammatory cytokine), and more IL-10 (anti-inflammatory cytokine) than RAW 264.7 peritoneal macrophages.

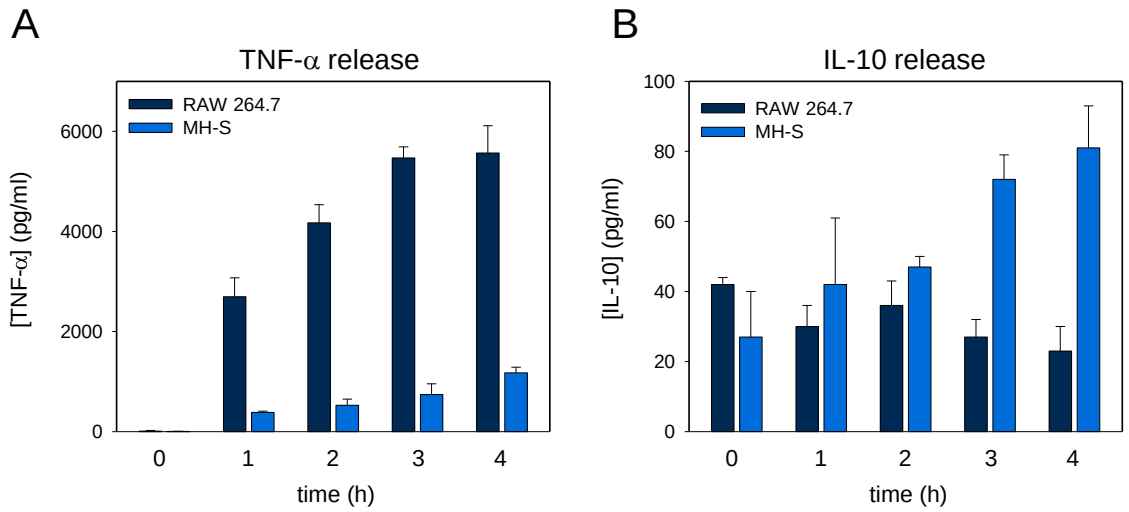


Figure 4: Time courses of TNF-α and IL-10 releases. MH-S and RAW 264.7 cells were stimulated with LPS (1 μg/ml) and culture supernatants were collected. TNF-α (A) or IL-10 (B) concentrations were determined by ELISA. Data are expressed as mean ± SE.

In summary, after LPS stimulation, RAW 264.7 cells produced more TNF-α and ROS than MH-S cells and the expression of CD14 was increased only in RAW 264.7 peritoneal macrophages. On the other hand, MH-S cells released more IL-10 than RAW 264.7 cells. These results indicate that the response to LPS is less pro-inflammatory in MH-S AMs than in RAW 264.7 peritoneal macrophages.

4.2 Characterization of synthetic surfactant vesicles

In order to study the biophysical properties of synthetic surfactant vesicles, we first performed a DLS analysis to determine the hydrodynamic diameters of the vesicles. The hydrodynamic diameters obtained for sPL and sSPC MLVs were higher than 3 μm (data not shown). We also analyzed the hydrodynamic diameters of synthetic surfactant SUVs. As we can see in figure 5A, the DLS scan of sPL SUVs showed two different peaks: one

corresponds to SUVs of 42 ± 3 nm and the other one to a small population of MLVs with a diameter of more than 3 μm. The scan of sSPC SUVs presented also two peaks: one corresponds to SUVs of 56 ± 1 nm and the other one to MLVs with a diameter bigger than 3 μm.

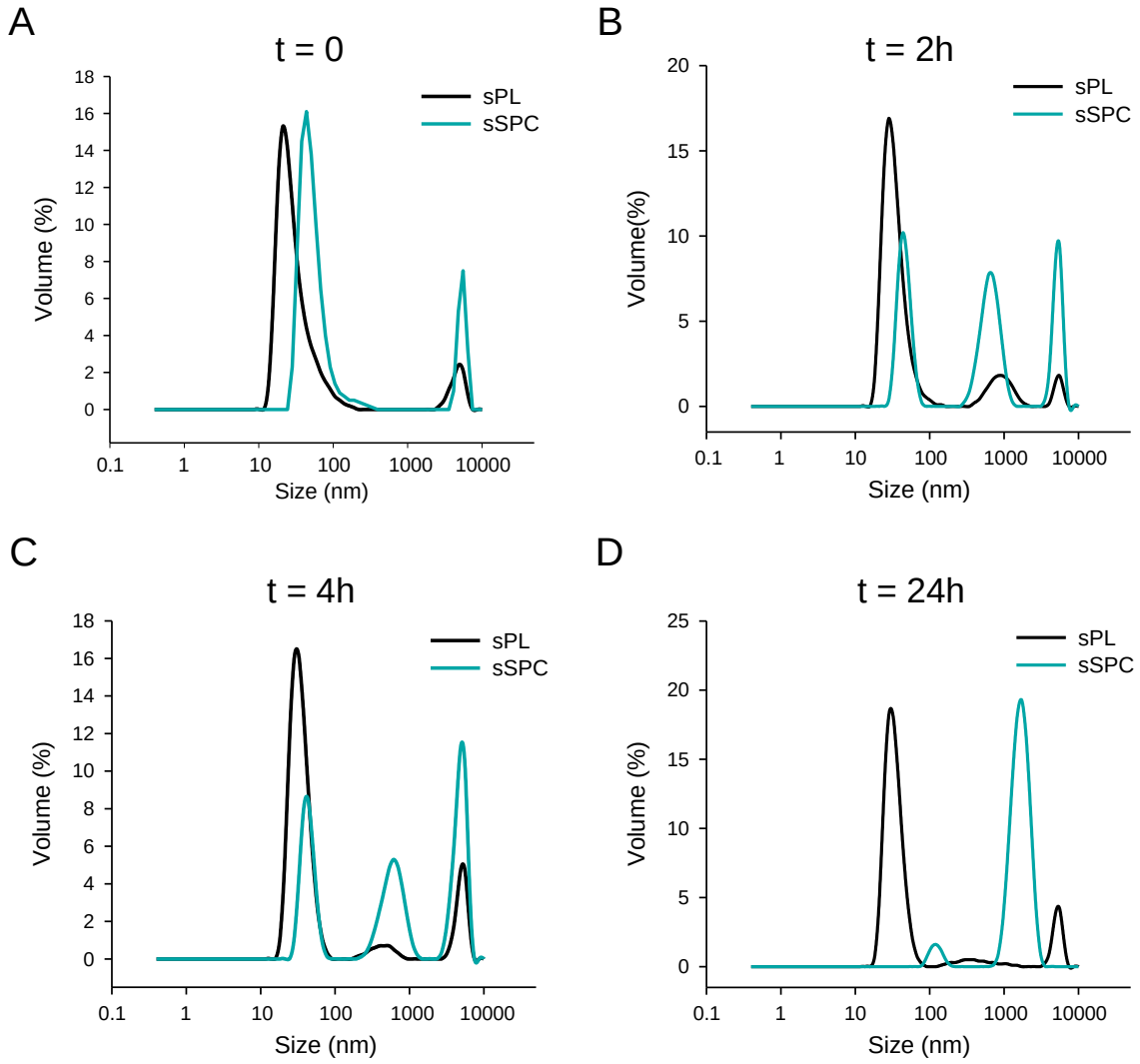


Figure 5: sPL SUV hydrodynamic diameter is stable for 24 hours, whereas sSPC SUVs tend to aggregate over time. sSPC and sPL SUVs were prepared at a final concentration of 250 μg/ml in 0.9% NaCl, and were incubated at 37ºC for 0, 2, 4 or 24 hours (A, B, C or D, respectively). Then, the hydrodynamic diameter of sPL or sSPC SUV preparations was determined by DLS at 25ºC. The *y* axis represents the volume percentage and the *x* axis denotes the hydrodynamic diameter of the particles present in the solution. A representative experiment of three different scans for each sample is here depicted.

To examine the stability of the vesicles over time we incubated sPL and sSPC SUVs for 2, 4, or 24 hours at 37ºC. DLS scans are represented in figures 5B, 5C and 5D. DLS studies revealed that sPL SUVs are almost stable over time, because after 24 hours at 37ºC 82.8% of the volume occupied by the particles in the solution corresponds to SUVs (it must be noted that in DLS scans the peak height or area is not only proportional to the amount of the particles in the solution, it is also proportional to their volume). However, sSPC SUVs tend to aggregate over time. After 24 hours at 37ºC, the smallest vesicles in the solution had a hydrodynamic diameter of 182.7 nm, which corresponds to unilamellar large vesicles. These results demonstrate that the presence of SP-C in synthetic surfactant vesicles promotes their aggregation. Taking this into consideration, in cellular experiments sSPC SUVs were added to cell cultures immediately after being sonicated, in order to avoid SP-C-induced vesicle aggregation.

To study the thermodynamic behavior of synthetic surfactant, we performed DSC scans of sPL and sSPC MLVs, which are depicted in figure 6. Both thermograms showed a single thermotropic event corresponding to the gel-to-liquid phase transition.

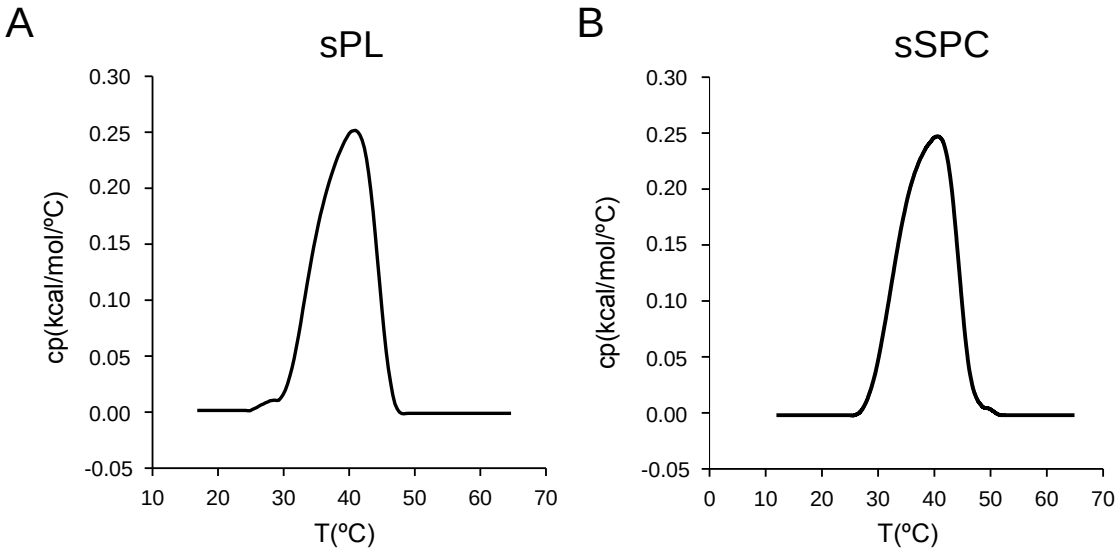


Figure 6: DSC heating scans of sPL and sSPC MLVs prepared in 150 mM NaCl and 5 mM Tris (pH 7.2) at a final concentration of 1 mg/ml. Calorimetric scans were performed at a rate of 0.5 ºC/min. Three consecutive scans were recorded for each sample. A representative scan of each sample is shown here.

Thermodynamic parameters calculated from DSC scans are summarized in table 1. sPL and sSPC MLV transitions presented similar melting temperatures (T_m) and similar transition enthalpies (ΔH). Both transitions were characterized by a broad peak with high similar temperature widths at half-height ($\Delta T_{1/2}$). This correlates with a small relative cooperativity of the process, which is generally the case of transitions observed with complex lipid mixtures (Cañadas and Casals 2013). These data allowed us to conclude that the presence of SP-C in synthetic surfactant membranes does not affect the thermodynamic properties of the phase transition. Moreover, from the shape of the thermogram peak, we could deduce that at 37ºC gel and liquid phases coexist in synthetic surfactant membranes.

Table 1: Thermodynamic data of sPL and sSPC MLVs (1 mg/ml in 150 mM NaCl, 5 mM Tris buffer, pH 7.2).

Sample	T_m (ºC)	$\Delta T_{½}$ (ºC)	ΔH (kcal/mol)
sPL	40.8 ± 0.2	12 ± 2	3 ± 1
sSPC	40.4 ± 0.4	11.9 ± 0.4	3.3 ± 0.6

Data are expressed as means ± standard deviation (SD) of three different scans.

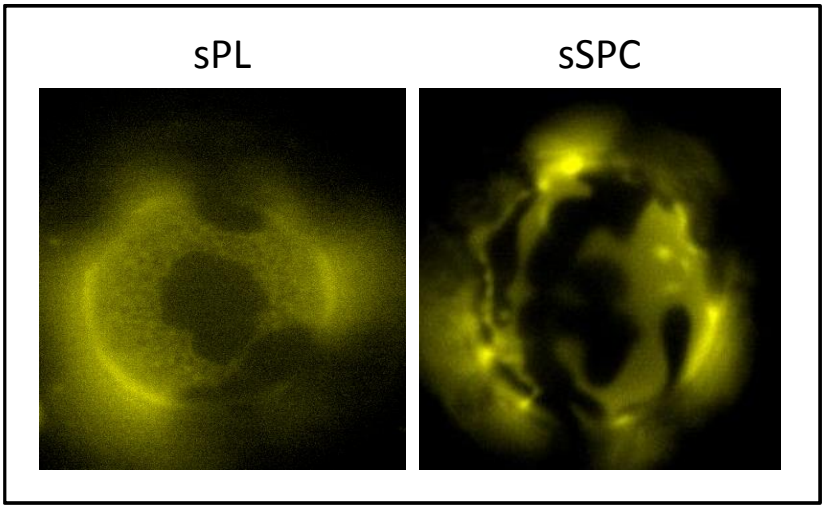


Figure 7: Fluorescence micrographs of sPL and sSPC GUVs stained with rhodamine-PE (in yellow) show ordered/disordered phase segregation. Micrographs are representative of two independent experiments.

The next step consisted in characterizing ordered/disordered phase segregation in synthetic surfactant membranes. We labeled sPL and sSPC GUVs with rhodamine-phosphatidylethanolamine (rhodamine-PE). This dye cannot enter gel phases, but it stains fluid domains. GUVs were observed by fluorescence microscopy (figure 7). As we can see in the micrographs, there was ordered/disordered phase segregation in both sPL and sSPC GUVs.

Finally, we measured sPL and sSPC SUV zeta potentials (table 2). sPL SUVs were negatively charged, due to the presence of anionic POPG and PA. Although the hydrophilic region of SP-C contains two positively charged residues (an arginine and a lysine) (Creuwels et al. 1995), its presence in sSPC vesicles did not affect their negative charge, which was similar to the one of sPL SUVs. This may be due to the small content of recombinant SP-C in sSPC membranes (only 2% by weight).

Table 2: Zeta potential of sPL and sSPC SUVs (1 mg/ml in 5mM Tris buffer, pH 7.2).

Sample	sPL	sSPC
Z potential (mV)	-37 ± 1	-40 ± 2

Data are expressed as means ± SD of three different scans.

4.3 Inhibition of LPS-induced macrophage stimulation

To determine whether synthetic surfactant vesicles were able to alter the response of AMs to LPS, we first analyzed the effect of sPL and sSPC SUVs on LPS-induced TNF-α mRNA production and release by MH-S murine AMs. Cells were stimulated with 1 μg/ml LPS in the presence or absence of 250 μg/ml sPL or sSPC SUVs. As it is shown in figure 8, synthetic surfactant SUVs significantly attenuated TNF-α mRNA production (figure 8A) and secretion (figure 8B). This inhibitory effect upon TNF-α release was increased in the absence of SP-C, and the reduction in mRNA production was similar for sPL and sSPC SUVs. Therefore, we concluded that the modulatory effect on TNF-α mRNA production and release is carried out by the lipid component of synthetic surfactant.

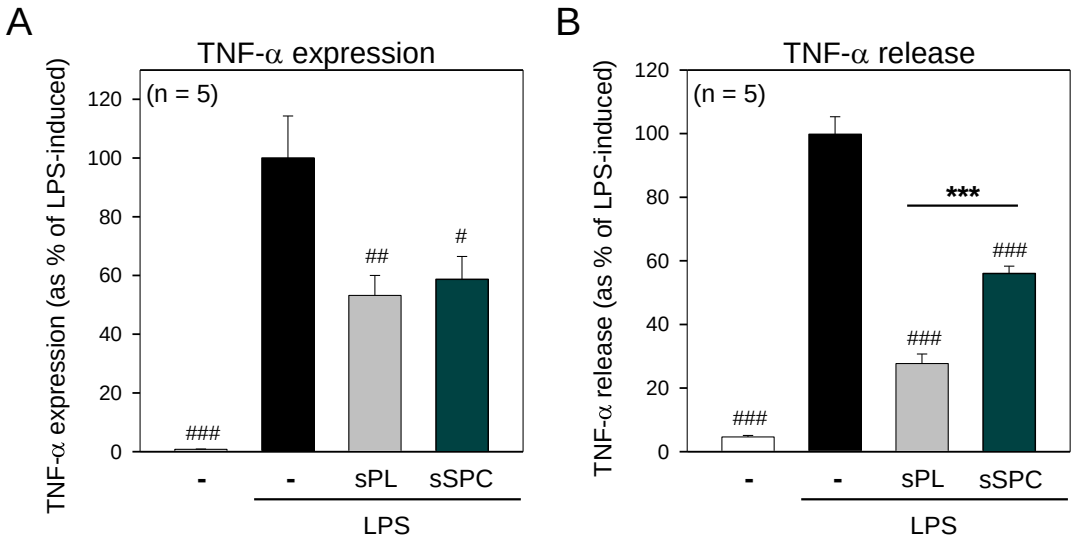


Figure 8: sPL and sSPC vesicles inhibit LPS-induced TNF-α mRNA production and release. MH-S cells were stimulated with LPS (1 μg/ml) in the presence or absence of sPL or sSPC SUVs (250 μg/ml) for 1 hour (A) or 4 hours (B). (A) TNF-α mRNA production was measured by qPCR. Data are expressed as percentages of LPS-induced TNF-α expression ± SE. (B) TNF-α release was measured by ELISA. Data are expressed as percentages of LPS-induced TNF-α release ± SE. Basal TNF-α secretion was 34 ± 2 pg/ml, and the average LPS-induced TNF-α release in the absence of surfactant was 584 ± 70 pg/ml. (***$p<0.001$ between indicated groups; #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs LPS).

Next, we analyzed if sPL or sSPC MLVs had the same inhibitory properties on LPS-elicited TNF-α secretion as synthetic surfactant SUVs. As we can see in figure 9, sPL MLVs inhibited TNF-α release to a lesser extent than sPL SUVs. However, there was no significant difference between the inhibitory effect of sSPC SUVs and MLVs, although the same tendency was observed. Interestingly, sPL and sSPC MLVs promoted similar decreases in TNF-α release, whereas sPL SUVs had a greater effect than sSPC SUVs (figure 8B).

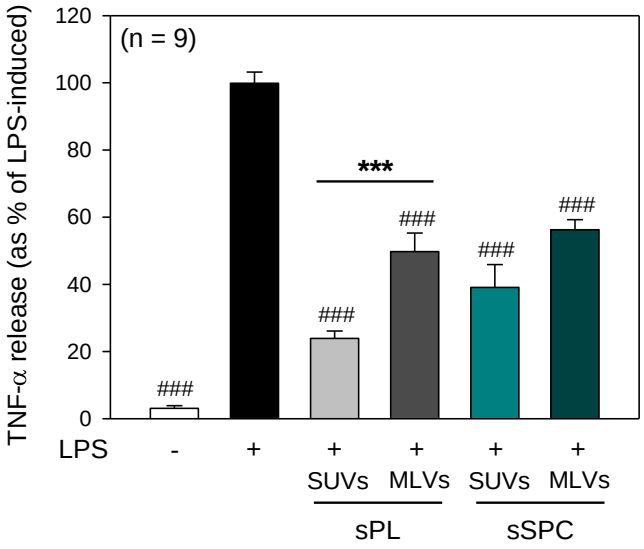


Figure 9: sPL and sSPC MLVs inhibit LPS-induced TNF-α release to a lesser extent than synthetic surfactant SUVs. MH-S cells were stimulated with LPS (1 μg/ml) in the presence or absence of sPL or sSPC MLVs or SUVs (250 μg/ml) for 4 hours. TNF-α release was measured by ELISA. Data are expressed as percentages of LPS-induced TNF-α release ± SE. (***$p<0.001$ between indicated groups; ###$p<0.001$ vs LPS).

The following step consisted in studying if synthetic surfactant immunoregulatory action was restricted to a single cell line, or was a general effect. We compared the effect of sPL and sSPC SUVs on TNF-α secretion after stimulation with LPS in MH-S and RAW 264.7 macrophages. As it is shown in figure 10, sPL SUVs were also able to diminish TNF-α release in RAW 264.7 cells, although the inhibition was significantly greater in MH-S AMs. This is probably due to the fact that RAW 264.7 peritoneal macrophages produce more TNF-α than MH-S cells (figure 4A). The same tendency was observed with sSPC SUVs, although their effect on RAW 264.7 cells was not significant. We can conclude that the immunomodulatory action of synthetic surfactant is not restricted to MH-S AMs.

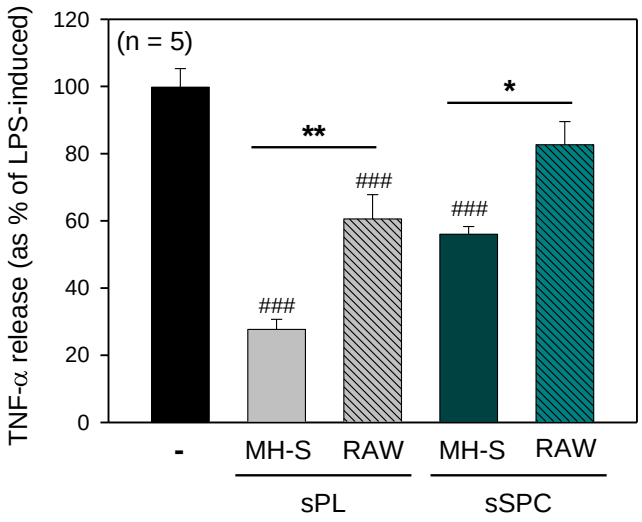


Figure 10: The inhibition of LPS-induced TNF-α release by surfactant SUVs is higher in MH-S cells than in RAW 264.7 peritoneal macrophages. MH-S and RAW 264.7 cells were stimulated with LPS (1 μg/ml) in the presence or absence of sPL or sSPC SUVs (250 μg/ml) for 4 hours. TNF-α secretion was measured by ELISA. Data are expressed as percentages of LPS-induced TNF-α release ± SE. (*p<0.05, **p<0.01 between indicated groups; ###p<0.001 vs LPS).

To determine whether surfactant inhibitory effect exclusively affected TNF-α, or if it was able to attenuate the production of other LPS-induced pro-inflammatory mediators, we examined the influence of sPL and sSPC SUVs upon iNOS mRNA and protein levels (figure 11). The lipid component of synthetic surfactant (sPL) decreased iNOS mRNA production and protein levels. Surprisingly, the presence of SP-C in sSPC SUVs promoted a significantly higher reduction in both parameters. Collectively, these results indicate that SP-C has a specific inhibitory effect on iNOS expression, whereas the lipid component of synthetic surfactant attenuates both TNF-α and iNOS productions.

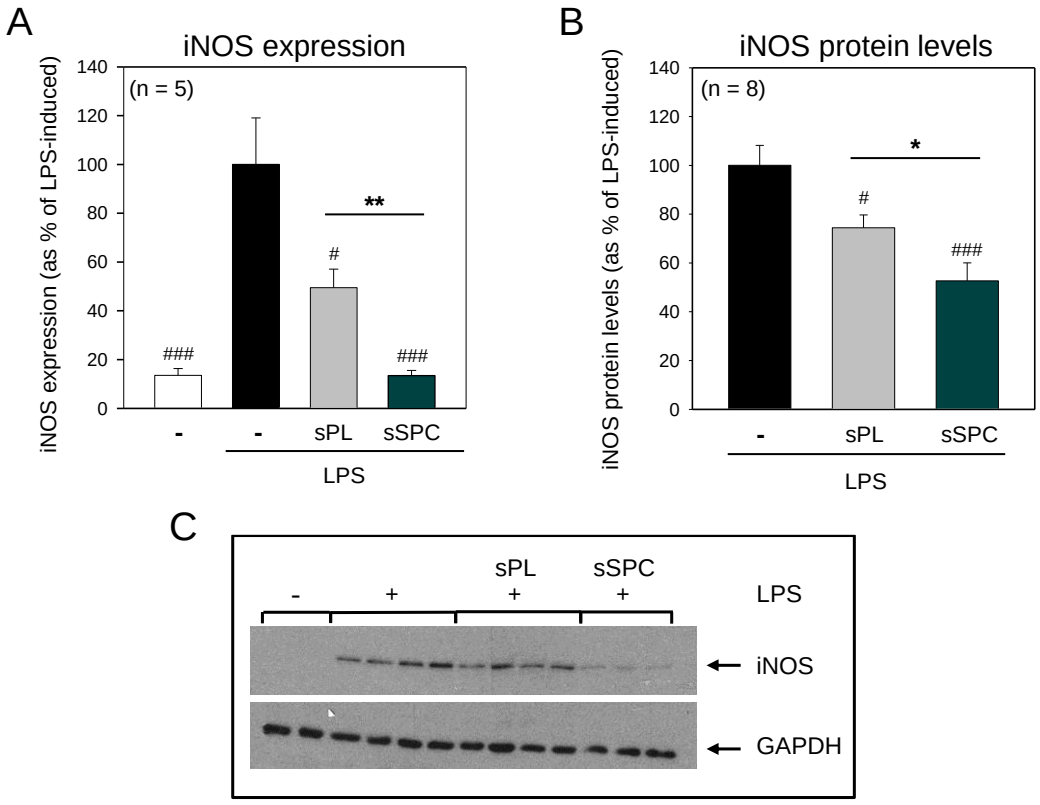


Figure 11: sPL and sSPC vesicles inhibit LPS-induced iNOS expression. MH-S cells were stimulated with LPS (1 μg/ml) in the presence or absence of sPL or sSPC SUVs (250 μg/ml) for 1 (A) or 6 (B) hours. (A) iNOS mRNA production was measured by qPCR. Data are expressed as percentages of LPS-induced iNOS expression ± SE. (B) iNOS protein levels were detected by western-blot and quantified by densitometry. LPS-induced iNOS protein level in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (C) Representative iNOS western-blot in MH-S cells treated as described in B. (*$p<0.05$, **$p<0.01$ between indicated groups; # $p<0.05$, ###$p<0.001$ vs LPS).

4.4 Inhibition of LPS-activated signaling pathways

When LPS binds to CD14 and TLR4/MD2 complex on AM surface, it triggers the activation of different signaling cascades. Among those are the pathways involving PI3K/Akt; ERK, JNK and p38 MAPKs; and NF-κB transcription factor. This leads to the induction of pro-inflammatory mediators such as TNF-α and iNOS (O'Toole and Peppelenbosch 2007; Bode et al. 2012).

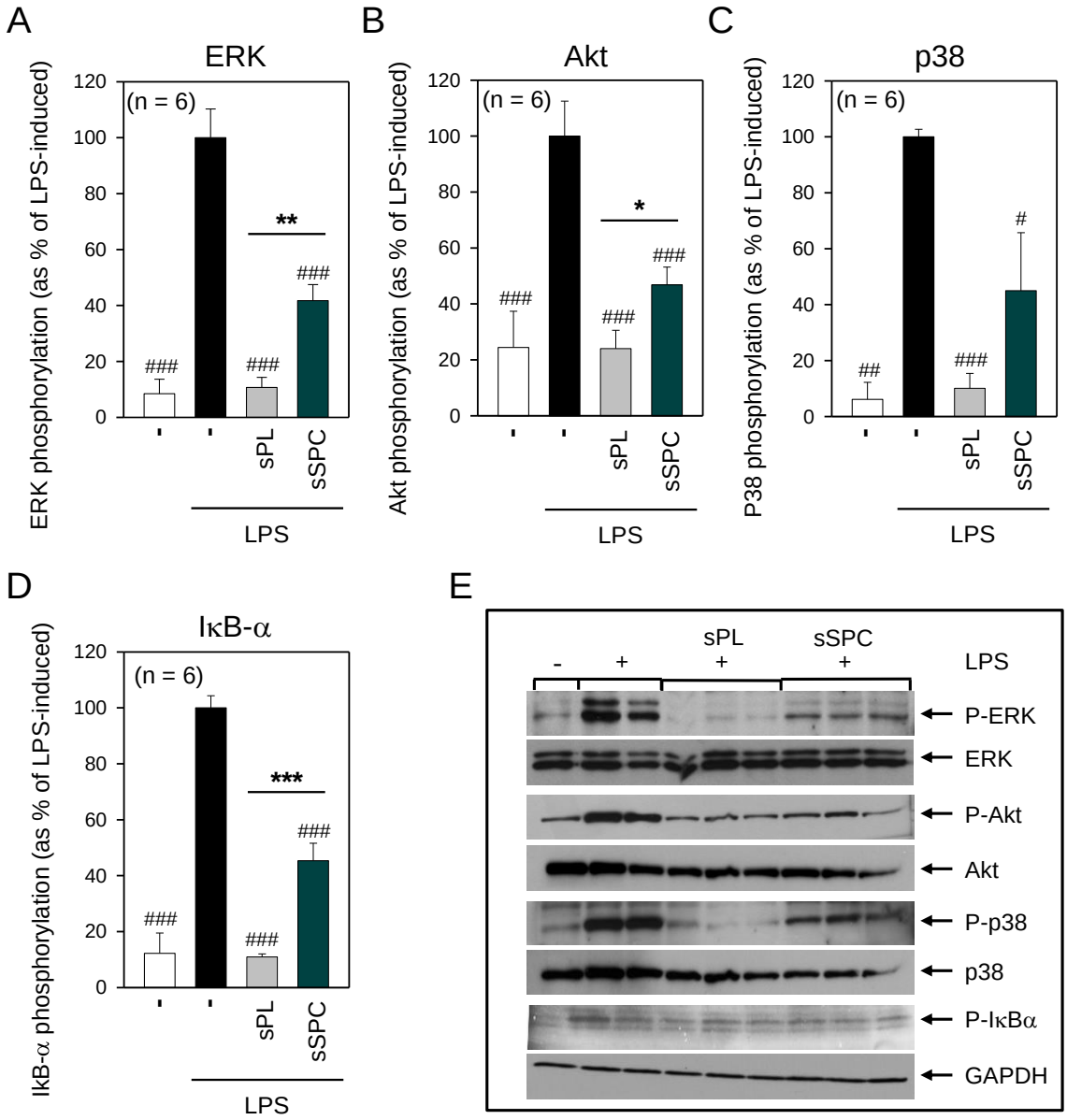


Figure 12: Synthetic surfactant SUVs inhibit LPS-induced signaling. MH-S cells were stimulated with LPS (1 μg/ml) in the presence or absence of sPL or sSPC SUVs (250 μg/ml) for 10 (A, B, C) or 30 (D) minutes. Then, ERK (A), Akt (B), p38 (C) and IκB-α (D) phosphorylations were detected by western-blot with phospho-specific antibodies, and quantified by densitometry using total protein or GAPDH as loading control. LPS-induced phosphorylation level in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (E) Representative western-blot images of MH-S cells treated as explained above. (*p<0.05, **p<0.01, ***p<0.001 between indicated groups; #p<0.05, ##p<0.01, ###p<0.001 vs LPS).

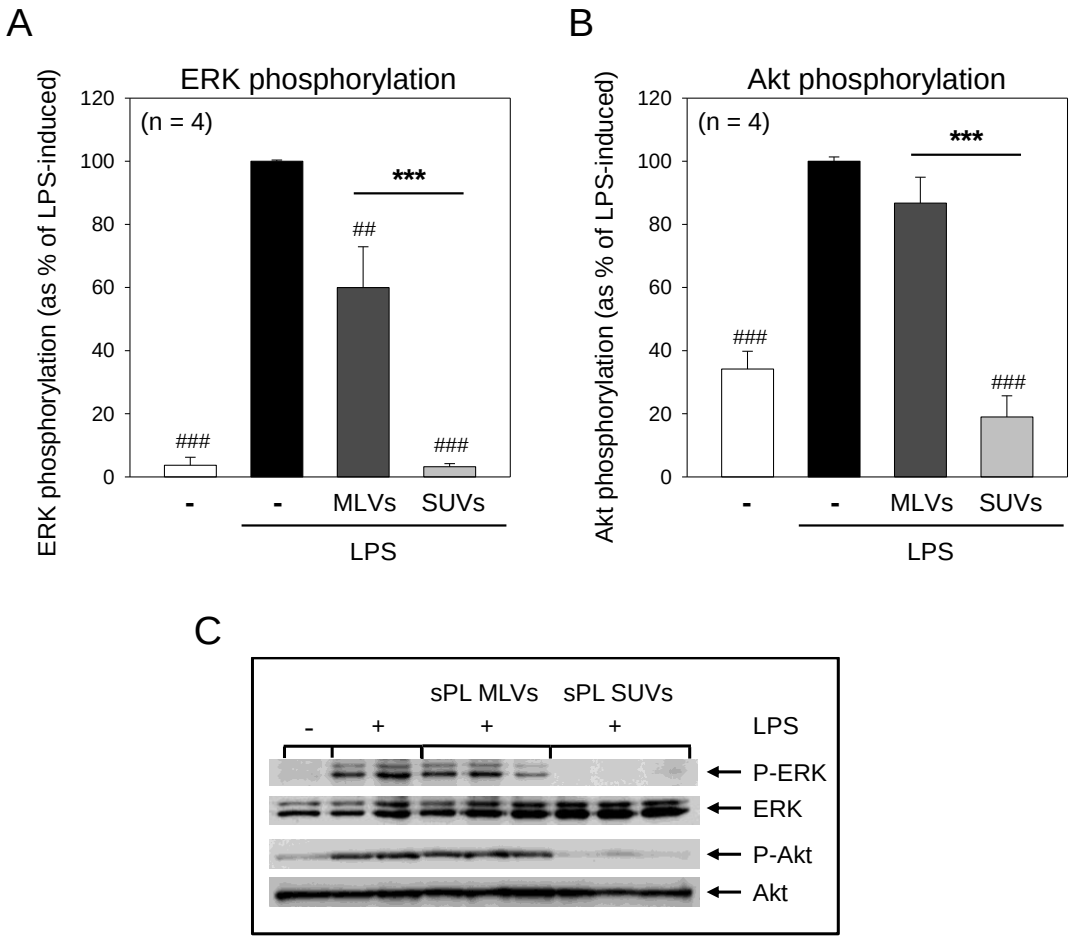


Figure 13: sPL MLVs attenuate LPS-induced signaling to a lesser extent than SUVs. MH-S cells were stimulated with LPS (1 μg/ml) in the presence or absence of sPL MLVs or SUVs (250 μg/ml) for 10 minutes. Then, ERK (A) and Akt (B) phosphorylations were detected by western-blot with phospho-specific antibodies, and quantified by densitometry using total protein as loading control. LPS-induced phosphorylation level in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (C) Representative western-blot images of MH-S cells treated as explained above. (***p<0.001 between indicated groups; ##p<0.01, ###p<0.001 vs LPS).

In our next experiments, we investigated the effect of synthetic surfactant on LPS-elicited phosphorylation of Akt, ERK, p38 and IκB-α, which is NF-κB inhibitor, and whose phosphorylation promotes its degradation, activating NF-κB. As it is shown in figure 12, stimulation of MH-S cells with LPS induced Akt, ERK, p38 and IκB-α phosphorylation. Treatment of the cells with sPL and sSPC SUVs in addition to LPS decreased the phosphorylation of these 4 proteins, and this inhibitory effect was greater in

the absence of SP-C. This result is consistent with TNF-α production data, and suggests that the lipid component of synthetic surfactant is responsible for the inhibition of LPS-signaling pathways.

In order to analyze if surfactant MLVs were able to modulate LPS-signaling pathway activation, we studied ERK and Akt phosphorylations after stimulating MH-S AMs in the presence or absence of sPL SUVs or MLVs (figure 13). LPS-induced phosphorylation of ERK and Akt was also inhibited by sPL MLVs, but the effect was lower than the one observed with sPL SUVs, accordingly with TNF-α release results.

4.5 Surfactant vesicle endocytosis

In the alveolar space, surfactant is internalized by AMs and type II pneumocytes by a mechanism that involves clathrin-mediated endocytosis accompanied by actin polymerization (Agassandian and Mallampalli 2013). In order to know whether synthetic surfactant anti-inflammatory effect takes place in the extracellular medium or not, we first analyzed if synthetic surfactant SUVs were endocytosed by MH-S AMs. For this purpose, MH-S macrophages were incubated with DiI-labeled sPL SUVs for 10 minutes, 30 minutes, one, 4 or 24 hours. Then, cells were fixed, nuclei were stained with DAPI and CD14 was detected with an FITC-conjugated antibody. As it is shown in confocal micrographs (figure 14), after incubating sPL SUVs with the cells for 10 minutes, most of the vesicles co-localized with CD14 at the cell membrane. However, after a 30 minute-long incubation, DiI-labeled sPL SUVs were observed inside the cells. We noticed that the amount of internalized sPL SUVs increased with the incubation time. Same results were obtained with sSPC SUVs (data not shown).

We next examined which pathway was involved in synthetic surfactant SUV endocytosis. We treated MH-S AMs for 30 minutes with different endocytosis inhibitors: 2.5 mM amantadine, 100 μM monodansylcadaverine, (both clathrin-dependent endocytosis inhibitors), 50 μg/ml nystatin (caveolae-dependent endocytosis inhibitor) (Ivanov 2008), 25 μM monensin (a ionophore that prevents endosomal maturation) (Mollenhauer et al. 1990) and 0.1 μM bafilomycin A1 (specific inhibitor of the vacuolar proton pump) (Gagliardi et al. 1999); prior to the addition of DiI-labeled sPL SUVs for 30 minutes. After that, cells were fixed and CD14 was detected with an FITC-conjugated antibody. Confocal

micrographs are depicted in figure 15. sPL endocytosis was almost suppressed by amantadine, and was also attenuated by monodansylcadaverine, both clathrin-mediated endocytosis inhibitors. This inhibition was also appreciable in the case of bafilomycin A1 and monensin (both of them general endocytosis disruptors), and to a lesser extent with nystatin, which is a caveolae-mediated endocytosis inhibitor. Once more, same findings were obtained with sSPC SUVs (data not shown).

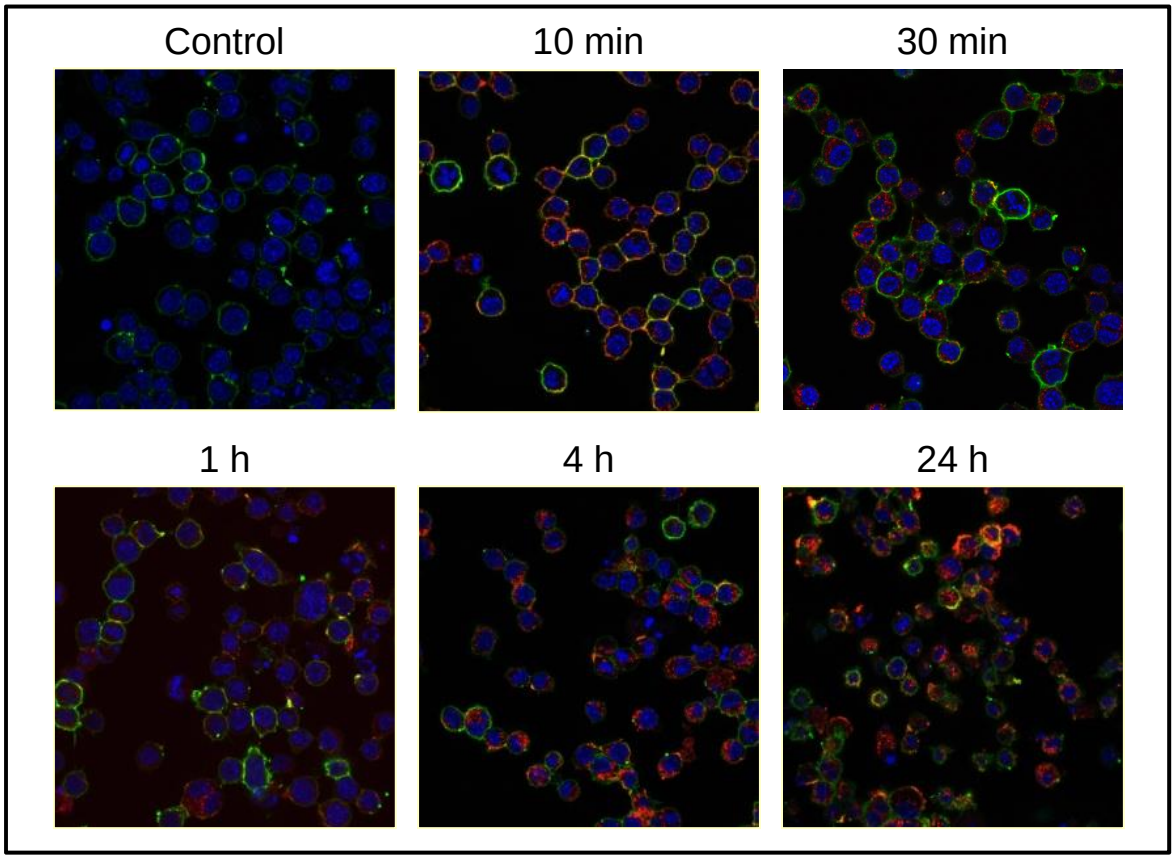


Figure 14: Surfactant phospholipids are internalized by MH-S cells. Macrophages were incubated with 250 μg/ml DiI-labeled sPL SUVs (in a 200:1 phospholipids:probe molar ratio) (in red) for 10 minutes, 30 minutes, 1, 4 or 24 hours. Then, cells were fixed, nuclei were stained with 1 μg/ml DAPI (in blue) and CD14 was detected with an FITC-conjugated antibody (in green). Confocal micrographs are representative of three independent experiments.

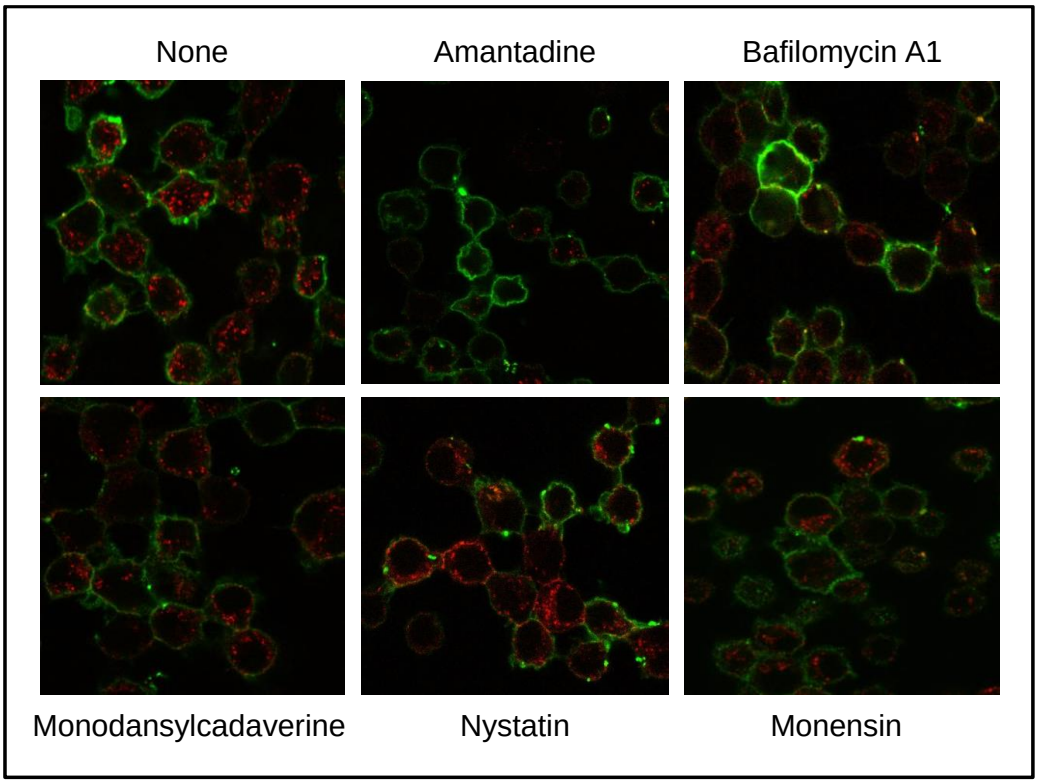


Figure 15: Endocytosis of synthetic surfactant SUVs by mouse alveolar macrophages is clathrin-dependent. MH-S cells were incubated with different endocytosis inhibitors for 30 minutes: 2.5 mM amantadine, 100 μM monodansylcadaverine, (both clathrin-dependent endocytosis inhibitors), 50 μg/ml nystatin (caveolae-dependent endocytosis inhibitor), 25 μM monensin (a ionophore that prevents endosomal maturation) and 0.1 μM bafilomycin A1 (specific inhibitor of the vacuolar proton pump), or none. Then, cells were incubated with 100 μg/ml DiI-labeled sPL SUVs (in a 200:1 phospholipids:probe molar ratio) (in red) for 30 minutes. CD14 was detected with an FITC-conjugated antibody (in green). Confocal micrographs are representative of three independent experiments.

Altogether, confocal microscopy analysis of surfactant endocytosis revealed that sPL and sSPC SUVs were endocytosed by MH-S AMs in a clathrin-dependent way. This endocytosis began almost ten minutes after the addition of the vesicles. If sPL and sSPC exert their immunomodulatory action in the extracellular medium, the modulation of LPS-response should disappear as surfactant vesicles are internalized. Our next step was analyzing the effect of increasing incubation times with sPL and sSPC SUVs prior to LPS addition on the immunoregulatory properties of synthetic surfactant vesicles.

4.6 Effect of an increasing preincubation time with surfactant vesicles

We measured TNF-α release of MH-S cells preincubated for 0, 10, 30 or 45 minutes with sPL SUVs before the stimulation with LPS (figure 16). As expected, the inhibition of TNF-α secretion was lower as the preincubation time with surfactant vesicles increased.

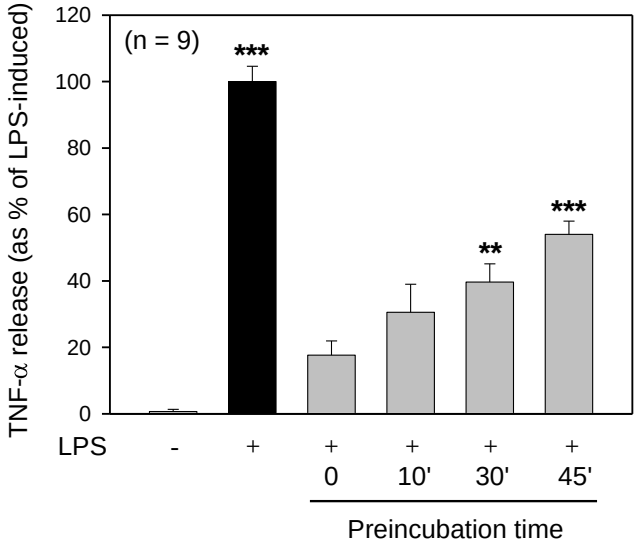


Figure 16: Increasing preincubation time with sPL SUVs decreases the inhibition of LPS-induced TNF-α release. MH-S cells were preincubated with sPL SUVs (250 μg/ml) for 0-45 minutes, before being stimulated with LPS (1 μg/ml) for 1 hour. TNF-α secretion was measured by ELISA. Data are expressed as percentages of LPS-induced TNF-α release ± SE. (**p<0.01, ***p<0.001 vs t = 0 preincubation time with sPL).

Furthermore, we analyzed ERK, p38, Akt and IκB-α phosphorylations after preincubating MH-S cells for 10 minutes, one or two hours with sPL SUVs prior to LPS addition. As controls, we also stimulated MH-S AMs with LPS in the presence or absence of sPL SUVs (figure 17). Accordingly with the results about TNF-α release (figure 16), increasing preincubation times with sPL SUVs reduced the inhibition of the phosphorylation of the 4 proteins. Same results were obtained with sSPC SUVs (data not shown).

Taking everything into consideration, we can conclude that surfactant vesicles exert their anti-inflammatory action on LPS-induced response in the extracellular medium, because their effect disappears as they are internalized by the cells.

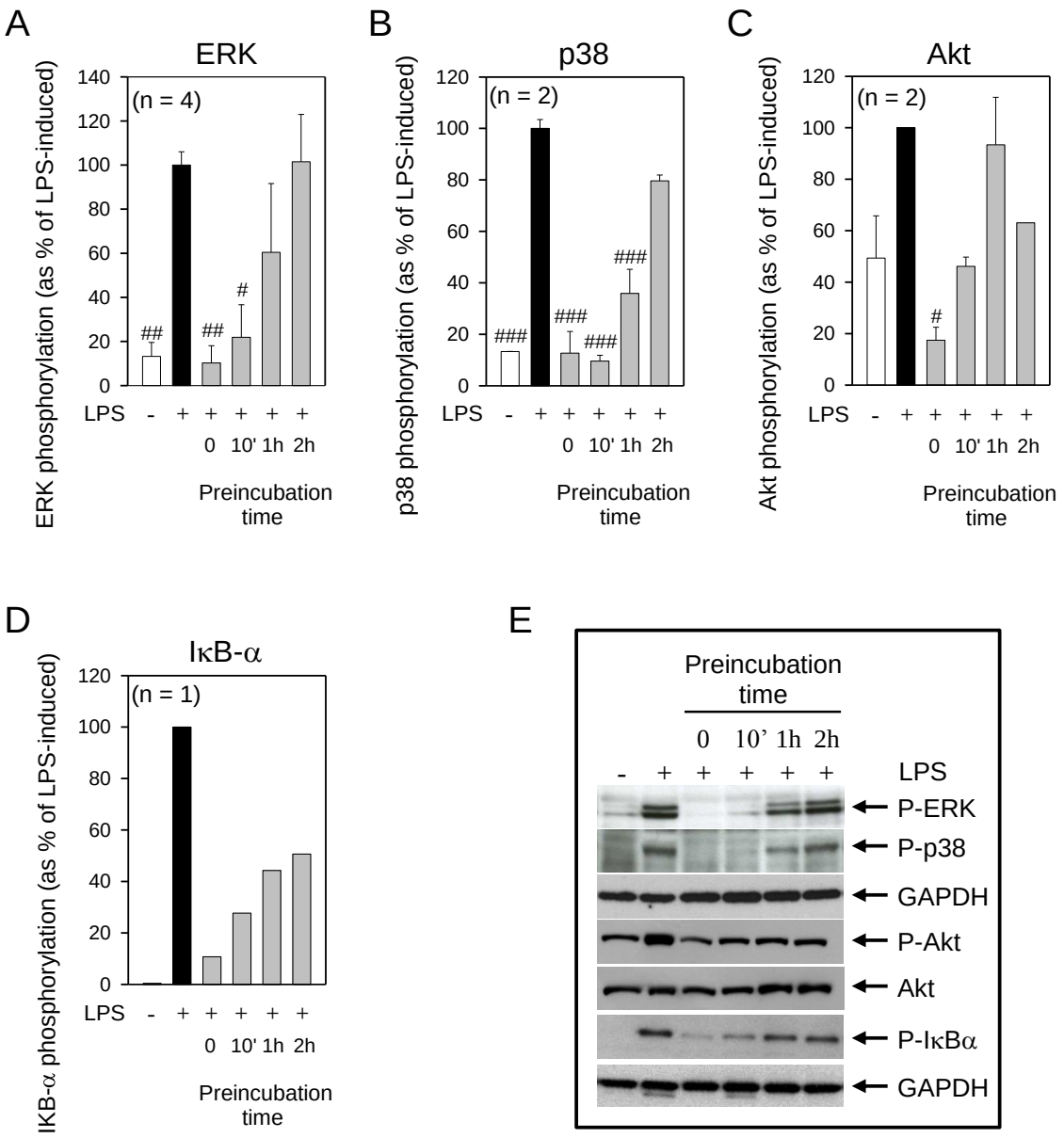


Figure 17: Inhibition of LPS-induced signaling disappears as the preincubation time with surfactant vesicles, prior to LPS addition, increases. MH-S cells were preincubated with sPL SUVs (250 μg/ml) for 0-2 hours, before being stimulated with LPS (1 μg/ml) for 10 (A, B, C) or 30 (D) minutes. Then, ERK (A), p38 (B), Akt (C) and IκB-α (D) phosphorylations were detected by western-blot with phospho-specific antibodies, and quantified by densitometry using total protein or GAPDH as loading control. LPS-induced phosphorylation level in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (E) Representative western-blot images of MH-S cells treated as explained above. (#p<0.05, ##p<0.01, ###p<0.001 vs LPS).

4.7 Inhibition of LPS-signaling is not caused by LPS trapping in surfactant vesicles

So far, we have demonstrated that surfactant vesicles inhibit LPS-induced response in AMs through an extracellular mechanism. We considered two possible explanations. The first one would be that surfactant membranes trap LPS molecules, preventing them from interacting with their membrane receptors in AMs. The second one would be that surfactant lipid component exerts its anti-inflammatory effect directly on the surface of AMs.

To examine whether synthetic surfactant membranes were able to bind to LPS molecules, 250 μg/ml sPL and sSPC MLVs were mixed with 1 μg/ml LPS in 0.9% sterile-filtered NaCl and incubated for 30 minutes with soft swelling at 37ºC. Mixtures were centrifuged to pellet surfactant vesicles and supernatant LPS concentration was determined.

Table 3: LPS endotoxin concentration in vesicle-free supernatants, after incubation in the absence or presence of sPL or sSPC MLVs.

	Mean EU/ml ± SE
Control + LPS	7173 ± 512
sPL + LPS	7652 ± 805
sSPC + LPS	8408 ± 1032

Data were obtained from 3 independent experiments.

As it is summarized in table 3, LPS concentrations did not vary between supernatants obtained from centrifugation of LPS alone and supernatants from samples that contained sPL or sSPC MLVs before centrifuging them. Thus, we concluded that sPL and sSPC are not able to trap LPS molecules and that surfactant vesicles exert their anti-inflammatory action directly on the surface of AMs.

4.8 Anti-inflammatory action of each synthetic surfactant lipid component

Among synthetic surfactant lipid components, DPPC and POPG have been shown to inhibit LPS-induced response. DPPC has been demonstrated to inhibit LPS-elicited IL-8 production in A549 lung epithelial cells by blocking TLR4 translocation into lipid raft

domains (Abate et al. 2010). Furthermore, it has been proved to modulate the biosynthesis of platelet-activating factor after LPS stimulation (Tonks et al. 2003), and to attenuate LPS-induced ROS production via downregulation of protein kinase C δ isoform (PKCδ) (Tonks et al. 2005) in a human monocytic cell line. On the other hand, POPG has been shown to disrupt LPS-signaling in primary rat and human AMs and in U937 cells, and to bind to CD14 and MD2 proteins (Kuronuma et al. 2009). In previous studies, POPG was also proved to inhibit the binding of LPS to LBP and CD14 (Hashimoto et al. 2003) and phosphatidylglycerol from egg was shown to bind to soluble LBP (Mueller et al. 2005).

Once we determined that sPL vesicles exerted their anti-inflammatory action directly on the surface of AMs, we wanted to analyze the contribution of each sPL lipid component to the inhibition of LPS-induced inflammatory response. For that purpose, we measured TNF-α release in MH-S AMs stimulated with LPS in the presence or absence of vesicles with different lipid compositions: DPPC, POPG, DPPC/PA (2.3:0.16 w/w), DPPC/POPG (2.3:1 w/w) and DPPC/POPG/PA (sPL) (2.3:1:0.16 w/w) (figure 18).

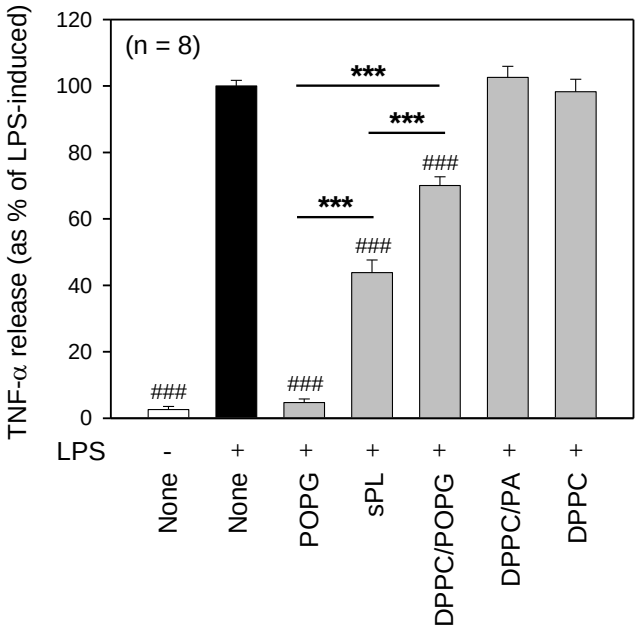


Figure 18: POPG-containing vesicles inhibit LPS-induced TNF-α secretion. MH-S cells were stimulated with LPS (1 μg/ml) in the presence or absence of 250 μg/ml POPG, sPL, DPPC/POPG, DPPC/PA or DPPC SUVs for 1 hour. TNF-α release was measured by ELISA. Data are expressed as percentages of LPS-induced TNF-α release ± SE. (***p<0.001 between indicated groups; ###p<0.001 vs LPS).

As we can see in figure 18, only POPG-containing vesicles were able to inhibit LPS-induced TNF-α release, whereas DPPC and DPPC/PA mixtures failed to show any modulatory effect. In fact, POPG vesicles promoted the greater inhibition, almost suppressing TNF-α secretion. Interestingly, DPPC/POPG/PA (sPL) SUVs attenuated TNF-α release to a significant greater extent than DPPC/POPG vesicles, even though the only difference between them is the small PA content (4.5% by weight). These results indicated that POPG is responsible for the inhibition of LPS-induced signaling in AMs, and that the presence of PA in DPPC/POPG/PA vesicles increases their anti-inflammatory action.

4.9 Physical characterization of different lipid vesicles

In order to understand why the presence of PA in sPL SUVs increases their anti-inflammatory effect, we decided to study the physical properties of DPPC, POPG, DPPC/PA (2.3:0.16 w/w), DPPC/POPG (2.3:1 w/w), DPPC/POPG/PA (sPL) (2.3:1:0.16 w/w) and sSPC vesicles.

First of all, Kuronuma et al. demonstrated that there was significant overlap between LPS and POPG binding sites in CD14. These regions interact with LPS negatively charged hydrophilic portion, so they suggested that POPG and other anionic phospholipids may compete with LPS by interfering with charge-dependent interactions with CD14 (Kuronuma et al. 2009). A more negative charge in the surface of the vesicles could improve the interaction between POPG and CD14. Considering that PA is also negatively charged, we measured the zeta potentials of the different lipid vesicles to determine whether the charge of the whole vesicle could influence its anti-inflammatory action. Results were summarized in table 4.

Table 4: Zeta potential of different lipid vesicles prepared at 1 mg/ml, in the presence of 0.22 mM $CaCl_2$.

Vesicles	POPG	sPL	DPPC/ POPG	sSPC	DPPC/ PA	DPPC
Z potential (mV)	-62 ± 5	-37 ± 1	-36 ± 1	-40 ± 2	-2.44 ± 0.04	7.5 ± 0.3

Data are expressed as means ± SD of three different scans.

Logically, POPG vesicles presented the most negative zeta potential. The presence of anionic compounds such as PA or POPG in lipid mixtures decreased their zeta potential. DPPC/PA vesicles had a slightly lower zeta potential than DPPC vesicles, but they both lacked to have a modulatory action on LPS-induced TNF-α release (figure 18) probably due to the absence of POPG. On the other hand, sPL and DPPC/POPG vesicles presented almost the same negative zeta potential, but the inhibitory effect of sPL SUVs was significantly greater than the one shown by DPPC/POPG SUVs (figure 18). These data proved that the presence of a high negative charge density in the surface of the vesicles is essential for their anti-inflammatory action but the difference between the inhibitory effect of sPL and DPPC/POPG vesicles on LPS-elicited TNF-α secretion cannot be explained by differences in zeta potential in their surface.

Next, we studied the thermodynamic behavior of DPPC, POPG, DPPC/PA, DPPC/POPG, DPPC/POPG/PA (sPL) and sSPC MLVs by DSC. Scans are depicted in figure 19 and thermodynamic parameters are summarized in table 5.

Table 5: Thermodynamic data and physical state of different lipid MLVs prepared at 1 mg/ml, in the presence of 0.22 mM $CaCl_2$.

Vesicles	POPG	sPL	DPPC/ POPG	sSPC	DPPC/ PA	DPPC
T_m (ºC)	-2*	40.8 ± 0.2	28 ± 1	40.4 ± 0.4	48.8 ± 0.5	41.57 ± 0.06
ΔH (kcal/mol)		3 ± 1	7 ± 2	3.3 ± 0.6	4.7 ± 0.7	4.2 ± 0.1
$\Delta T_{½}$ (ºC)		12 ± 2	11.1 ± 0.2	11.9 ± 0.4	6.34 ± 0.05	0.47 ± 0.03
Physical state at 37ºC	L_α*	L_α/L_β #	L_α (with ↓↓↓L_β) #	L_α/L_β #	L_β	$P_{\beta'}$

Numeric data are expressed as mean ± SD. * Data obtained from Avanti Polar Lipids. # Data confirmed by observation of rhodamine-PE-labeled GUVs by fluorescence microscopy.

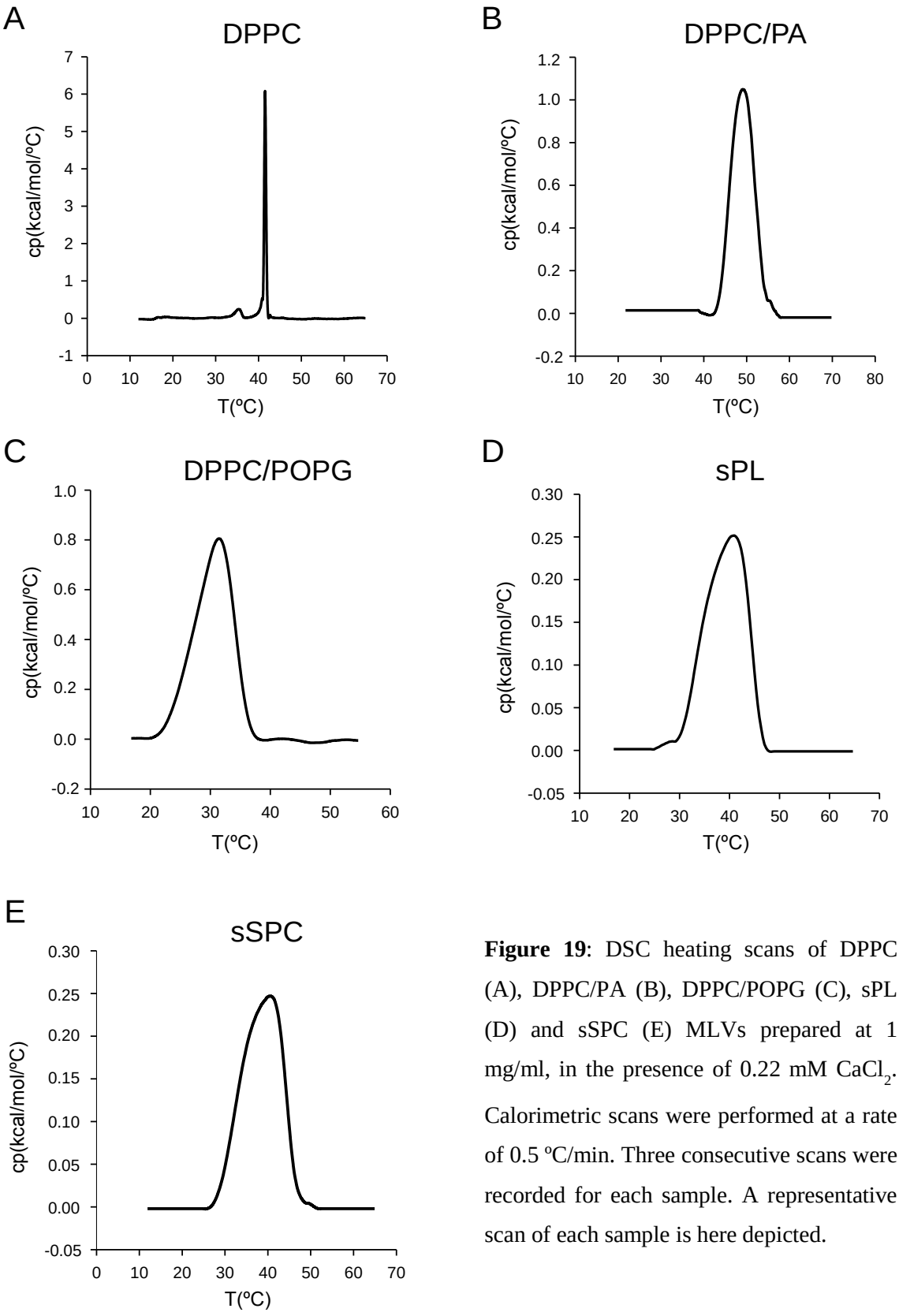


Figure 19: DSC heating scans of DPPC (A), DPPC/PA (B), DPPC/POPG (C), sPL (D) and sSPC (E) MLVs prepared at 1 mg/ml, in the presence of 0.22 mM $CaCl_2$. Calorimetric scans were performed at a rate of 0.5 ºC/min. Three consecutive scans were recorded for each sample. A representative scan of each sample is here depicted.

Figure 19A shows the typical DPPC DSC scan, exhibiting two transitions: a pretransition from the gel ($L_{\beta'}$) to the ripple ($P_{\beta'}$) phase, and the main transition from the ripple to the liquid-crystalline (L_{α}) phase with a melting temperature of 41.57 ± 0.06ºC and a small temperature width at half-height (Casals and Cañadas 2012). POPG thermogram could not be performed because the transition takes place at too low temperatures, but the value of the phase transition temperature was obtained from the supplier's information datasheet. DPPC/PA, DPPC/POPG, sPL and sSPC thermograms showed a single thermotropic event corresponding to the gel-to-liquid phase transition (figures 19B, 19C, 19D and 19E). As we can see in table 5, the temperature widths at half-height increased with the complexity of the lipid mixtures and the presence of PA or DPPC incremented the values of the melting temperatures and therefore the rigidity of the vesicles. Importantly, from the shape of the DSC peaks and the values of the phase transition temperatures, we deduced that at 37ºC DPPC and DPPC/PA vesicles were in the ripple and gel state respectively, POPG vesicles were in the liquid-crystalline phase, DPPC/POPG vesicles were at the end of the melting process (mainly in fluid state), and sPL and sSPC vesicles presented L_{α}/L_{β} phase coexistence. These results indicated that the fact that PA enhances sPL SUV anti-inflammatory action in comparison with DPPC/POPG SUVs could be due to PA-induced increment of the rigidity of the vesicles.

4.10 Palmitic acid-increased ordered/disordered phase segregation

As POPG interaction with CD14 is supposed to be charge-dependent, we hypothesized that PA could be inducing a re-organization of POPG molecules in sPL SUVs, increasing negative charge density in some regions, and favoring the binding of POPG to CD14. To ascertain it, we observed rhodamine-PE-labeled DPPC/POPG/PA and DPPC/POPG GUVs by fluorescence microscopy. As we can see in the micrographs (figure 20), DPPC/POPG vesicles presented small unstained domains, which correspond to ordered domains. However, DPPC/POPG/PA GUVs contained larger ordered regions. This observation indicated that PA reduces the miscibility between DPPC and POPG and increases ordered/disordered phase segregation in DPPC/POPG/PA vesicles, enriching fluid domains with POPG and ordered domains with DPPC. Therefore, the density of negative charges in fluid domains is likely to be augmented in DPPC/POPG/PA vesicles. These results allowed

us to conclude that PA-induced increment in L_α/L_β phase segregation in DPPC/POPG/PA vesicles may be responsible for the increased anti-inflammatory action of sPL vesicles in comparison to DPPC/POPG SUVs on the response of AMs to LPS.

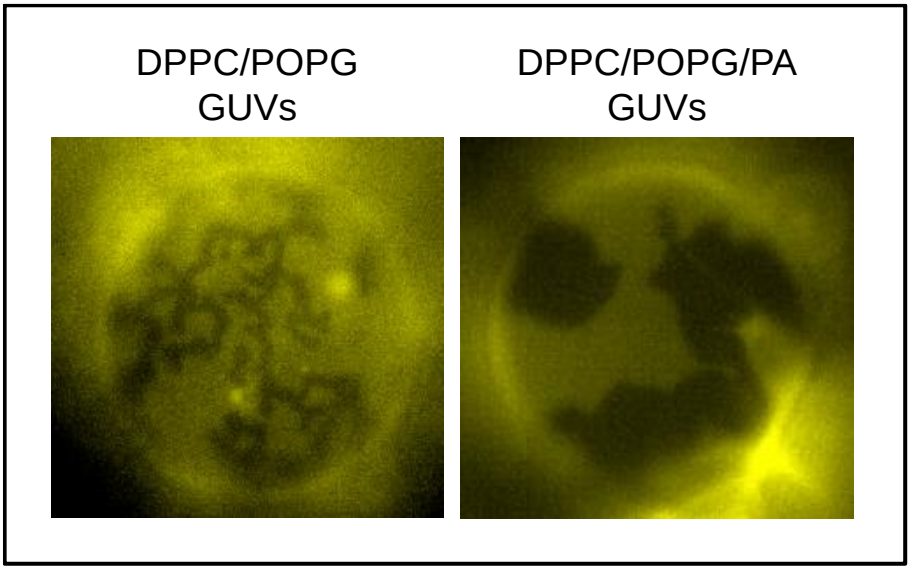


Figure 20: Palmitic acid increases ordered/disordered domain segregation in DPPC/POPG/PA vesicles. DPPC/POPG and DPPC/POPG/PA GUVs stained with rhodamine-PE (in yellow) were analyzed by fluorescence microscopy. Non-fluorescent domains in DPPC/POPG vesicles are smaller than in DPPC/POPG/PA vesicles. Micrographs are representative of two independent experiments.

5. DISCUSSION

In this chapter, we have studied the anti-inflammatory effect of a synthetic surfactant composed of DPPC, POPG, PA and recombinant human SP-C on LPS-induced response in MH-S murine AMs. sSPC was demonstrated to attenuate LPS-elicited ERK, p38, Akt and IκB-α phosphorylations; inhibiting the three key signaling pathways activated by LPS (MAPK, PI3K and NF-κB cascades) (figure 12). Furthermore, LPS-induced TNF-α and iNOS productions were also diminished by synthetic surfactant vesicles (figures 8 and 11). This immunomodulatory action is not restricted to MH-S AMs, since sPL and sSPC SUVs were also shown to attenuate TNF-α secretion in RAW 264.7 mouse peritoneal macrophages (figure 10). Previous studies have proved the anti-inflammatory action of this

synthetic surfactant in patients suffering ARDS (Spragg et al. 2003) and on LPS-challenged human monocytic cells (Wemhöner et al. 2009). However, the role of each component and their mechanism had not been analyzed yet.

Our results provide evidence that the lipid component of synthetic surfactant is responsible for the anti-inflammatory action on the response of AMs to LPS. sPL contributed to LPS-elicited iNOS production decrease (figure 11), TNF-α mRNA levels were modulated to the same extent by sPL and sSPC SUVs (figure 8A), and the inhibitions of LPS-induced TNF-α release (figure 8B) and signaling pathway activation (figure 12) were significantly higher in the absence of SP-C.

In these experiments, surfactant vesicles were added to the cells simultaneously with LPS. As all the signaling pathways activated by LPS were inhibited by synthetic surfactant vesicles, we hypothesized that sPL and sSPC might be exerting their anti-inflammatory action in the extracellular medium, either by trapping LPS molecules, or by interfering with LPS binding to the surface of AMs. Confocal microscopy analysis proved that synthetic surfactant SUVs were endocytosed by AMs by a clathrin-dependent mechanism (figure 14 and 15). This endocytosis began almost ten minutes after the addition of the vesicles, and increased as the incubation time augmented. This finding was consistent with previous knowledge about surfactant metabolism in the alveolar space, where it is internalized by AMs and type II pneumocytes by a mechanism that involves clathrin-mediated endocytosis accompanied by actin polymerization, in order to assure its degradation and recycling (Agassandian and Mallampalli 2013). Experiments in which surfactant SUVs were preincubated with MH-S cells for increasing periods of time prior to the addition of LPS demonstrated that surfactant inhibitory action on LPS-elicited TNF-α release and signaling pathway activation decreased as the vesicles were internalized by AMs (figures 16 and 17), providing further evidence that synthetic surfactant vesicles exerted their anti-inflammatory effect in the extracellular medium.

We also performed an interaction assay between surfactant MLVs and LPS, which proved that sPL vesicles were not able to trap LPS molecules (table 3). Kuronuma et al. had previously conducted similar binding assays showing that POPG liposomes failed to remove LPS molecules from the solution in which both had been previously incubated (Kuronuma et al. 2009). Altogether, these results allowed us to conclude that synthetic

surfactant vesicles carried out their immunoregulatory action directly on the surface of AMs, probably by interfering with LPS binding to the cells.

Among sPL lipid components, POPG was found to be responsible for the attenuation of LPS-induced signaling in AMs, because only POPG-containing vesicles were able to decrease LPS-elicited TNF-α secretion, whereas DPPC and DPPC/PA mixtures failed to show any modulatory effect (figure 18). Interestingly, the presence of PA in DPPC/POPG/PA (sPL) vesicles increased their anti-inflammatory action, as DPPC/POPG/PA SUVs attenuated TNF-α release to a significant greater extent than DPPC/POPG vesicles, even though the only difference between them is the small PA content (4.5% by weight).

POPG immunoregulatory properties have already been described. Segregated pools of POPG have been shown to suppress respiratory syncytial virus and influenza A virus infections (Numata et al. 2010; Numata et al. 2012a), to disrupt TLR2 ligand-induced pro-inflammatory responses (Kandasamy et al. 2011) and to inhibit TNF-α and nitric oxide productions as well as activation of MAPK and NF-κB pathways induced by LPS in primary rat and human AMs and in U937 cells, by binding to CD14 and MD2 proteins (Kuronuma et al. 2009). In previous studies, POPG was also proved to inhibit the binding of LPS to LBP and CD14 (Hashimoto et al. 2003) and phosphatidylglycerol from egg was shown to bind to soluble LBP (Mueller et al. 2005). In this study, we have demonstrated that POPG is also able to regulate LPS-elicited activation of the PI3K/Akt pathway (figures 12 and 13). Synthetic surfactant POPG interaction with CD14, MD2 or LBP would be consistent with all our previous results, as it would promote the inhibition of all LPS-activated signaling pathways through an extracellular mechanism.

Zeta potential measurements proved that the presence of a high negative charge density on the surface of the vesicles is essential for their anti-inflammatory action (table 4). This finding is consistent with Kuronuma et al. previous work, in which they demonstrated that there was significant overlap between LPS and POPG binding sites in CD14. These regions interact with LPS negatively charged hydrophilic portion, so they suggested that POPG and other anionic phospholipids may compete with LPS by interfering with charge-dependent interactions with CD14 (Kuronuma et al. 2009). A

higher negative charge density on the surface of the vesicles could improve the interaction between POPG and CD14.

However, the difference between the inhibitory action of sPL and DPPC/POPG vesicles on LPS-elicited TNF-α secretion was not caused by a different zeta potential in the surface of the vesicles (table 4). DSC analysis showed that at 37ºC DPPC/POPG vesicles are at the end of the melting process, mainly in liquid-crystalline phase, whereas sPL and sSPC vesicles presented gel and fluid phase coexistence (figure 19 and table 5). Phase coexistence has been proved to exist also in human pulmonary surfactant membranes (Sáenz et al. 2006) and to be essential for surfactant biophysical functions (Casals and Cañadas 2012). These results indicated that the fact that PA enhances sPL SUV anti-inflammatory action in comparison with DPPC/POPG SUVs could be due to PA-induced increment of the rigidity of the vesicles.

Observation of rhodamine-PE-labeled DPPC/POPG GUVs revealed that these vesicles presented small unstained solid domains (figure 20). This high level of miscibility between DPPC and POPG has already been reported in previous studies (Ma et al. 1998; Sáenz et al. 2006). On the other hand, DPPC/POPG/PA (sPL) GUVs contained larger ordered domains rich in DPPC, indicating that the presence of PA in these vesicles reduced the miscibility between DPPC and POPG and increased ordered/disordered phase segregation, enriching fluid domains with POPG and ordered domains with DPPC, accordingly with previous results published by our group (Sáenz et al. 2006). Therefore, the density of negative charges in fluid domains is likely to be increased in DPPC/POPG/PA vesicles, favoring the binding of POPG to CD14. These results allowed us to conclude that PA-induced increment in L_α/L_β phase segregation in DPPC/POPG/PA vesicles may be responsible for the enhanced anti-inflammatory action of sPL vesicles in comparison with DPPC/POPG SUVs on the response of AMs to LPS.

In this study, DPPC did not exert any modulatory effect on LPS-induced response in AMs. However, DPPC has been demonstrated to inhibit LPS-elicited IL-8 production in A549 lung epithelial cells by blocking TLR4 translocation into lipid raft domains (Abate et al. 2010). Furthermore, it has been proved to modulate the biosynthesis of platelet-activating factor after LPS stimulation (Tonks et al. 2003), and to attenuate LPS-induced ROS production via downregulation of PKCδ isoform (Tonks et al. 2005) in a human

monocytic cell line. Interestingly, in these studies the maximum effect was observed after preincubating DPPC with the cells for 2 hours, and DPPC immunoregulatory properties appeared to be dependent on its uptake by the cells. This suggests that synthetic surfactant components like POPG or DPPC could have different effects depending on their location.

Synthetic surfactant MLVs were shown to have less immunoregulatory properties than SUVs on the response to LPS (figures 9 and 13). This is probably due to the fact that POPG molecules in the inner bilayers of MLVs are not exposed to the extracellular medium. This is physiologically relevant since exogenous surfactants are delivered to patients in the form of MLVs in order to mimic the structure of large surfactant aggregates, which have high surface activity and adsorb very rapidly to the air-liquid interface. Nevertheless, surface compression and expansion cycles generate small vesicles that have poor surface activity and are endocytosed by AMs (Casals and Cañadas 2012). Therefore, administration of this synthetic surfactant as MLVs would improve lung function preserving its anti-inflammatory properties on LPS-induced inflammation.

Concerning SP-C, sSPC SUVs were shown to have less inhibitory properties on LPS-induced response than sPL SUVs (figures 8B and 12). sSPC and sPL vesicles were proved to have same physical state, with ordered/disordered phase segregation at 37ºC (figures 6 and 7 and table 1), and same negative zeta potential (table 2), probably because of the small amount of SP-C in sSPC membranes (only 2% by weight). However, as it was observed in DLS analysis, the presence of SP-C in surfactant vesicles promotes their aggregation (figure 5). Actually, SP-C N-terminal segment has already been demonstrated to induce phospholipid vesicle aggregation (Plasencia et al. 2004). Even though we do not know the structure of these aggregates, POPG molecules may be less exposed to the extracellular medium in sSPC aggregated vesicles than in sPL SUVs, which would prevent them from exerting their anti-inflammatory action. Consistent with this rationale, there is no significant difference in the modulation of LPS-induced TNF-α release carried out by sSPC SUVs and MLVs (figure 9), as sSPC SUVs may have aggregated and may have functioned as sSPC MLVs.

Murine, porcine and rabbit SP-C have been shown to bind to the lipid A region of LPS and to CD14 (Augusto et al. 2002; Augusto et al 2003a), and SP-C inserted in DPPC vesicles has been proved to inhibit LPS-induced TNF-α production in peritoneal and

alveolar macrophages, presumably by trapping LPS molecules and preventing them from triggering their inflammatory action (Augusto et al. 2003b). Nonetheless, our results demonstrated that sSPC MLVs were not able to bind to LPS (table 3). In the experiments conducted by Augusto et al., the amount of SP-C inserted in DPPC vesicles was 10% by weight (Augusto et al. 2003b), whereas sSPC membranes contain only 2% of SP-C by weight. Moreover, synthetic surfactant vesicles contain POPG and PA in addition to DPPC. The hydrophilic region of SP-C contains two positively charged residues (an arginine and a lysine) (Creuwels et al. 1995). Augusto et al. suggested that these positive charges are involved in the interaction of SP-C with LPS (Augusto et al. 2002). However, it has been reported that SP-C distributes preferentially in fluid regions of surfactant bilayers and surfactant-like lipid mixtures (Nag et al. 1996; Bernardino de la Serna et al. 2004). In sSPC membranes, fluid regions are rich in POPG, which means that the density of negative charges in these domains could impede the interaction between SP-C and negatively charged LPS molecules.

On the other hand, recombinant human SP-C present in synthetic surfactant SUVs specifically modulated LPS-elicited production of iNOS, at both mRNA and protein levels (figure 11). Since SP-C did not specifically regulate neither LPS-induced activation of signaling pathways nor TNF-α secretion (figures 8 and 12), we can deduce that it must be exerting its anti-inflammatory action through a mechanism involving signaling pathways and transcription factors activated by LPS-stimulation which regulate iNOS expression but not TNF-α production. This excludes NF-κB, activating protein 1 (AP1), cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) or CCAAT-enhancer box binding protein (c/EBP) transcription factors, which control both TNF-α and iNOS transcription (Pautz et al. 2010; Bode et al. 2012). Studies with bacterial LPS showed that full transcriptional induction of iNOS and nitric oxide production only occurs after type I interferon synthesis and signaling through the Janus kinase (JAK)-signal transducer and activator of transcription 1α (STAT1α) pathway (Gao et al. 1997; Gao et al. 1998). In macrophages, JAK3 inhibition resulted in lower STAT1 activation and iNOS production, but had no effect on TNF-α levels (Sareila et al. 2008). Further studies must be conducted in order to elucidate if SP-C is able to regulate JAK-STAT signaling cascade in AMs.

In conclusion, our study provides new insights about the influence of each synthetic surfactant component on LPS-induced response in AMs. We have demonstrated that POPG is responsible for the extracellular inhibition of LPS-elicited pro-inflammatory signaling, reinforcing the importance of its presence in exogenous surfactant preparations. Furthermore, ordered/disordered phase segregation in surfactant membranes has been proved to be essential for improving synthetic surfactant immunoregulatory properties. Herein, PA or other compounds that induce the enrichment of POPG in segregated domains should also be included in synthetic surfactant formulations. In fact, PA is a common additive in replacement surfactants because it increases their surface activity (Sáenz et al. 2006; Mingarro et al. 2008). The anti-inflammatory and biophysical properties of this synthetic surfactant based on recombinant human SP-C raise its use as a promising therapy to counteract LPS or Gram-negative bacteria-induced inflammation, especially in situations in which lung function is compromised.

CHAPTER 2

Internalized surfactant vesicles modify the activation state of alveolar macrophages in the presence or absence of bacterial lipopolysaccharide

1. ABSTRACT

Pulmonary surfactant phospholipids and proteins have been shown to regulate the immune response of alveolar macrophages (AMs). However, little is known about their immunomodulatory action once they have been endocytosed by these cells. The aim of this work was to analyze if internalized components of a synthetic surfactant based on recombinant human surfactant protein C (SP-C) were able to modulate the activation state of MH-S murine AMs stimulated with bacterial lipopolysaccharide (LPS). Internalized synthetic surfactant lipids showed no regulatory effect on the activation of mitogen-activated protein kinases (MAPKs), but they inhibited LPS-induced activation of the phosphatidylinositol-3-kinase (PI3K) and nuclear factor-κB (NF-κB) pathways. Consequently, they reduced Akt phosphorylation and promoted glycogen synthase kinase 3 (GSK3) activation. Moreover, they decreased LPS-elicited tumor necrosis factor α (TNF-α) (pro-inflammatory cytokine) production and interleukin (IL)-10 (anti-inflammatory cytokine) release. Our results also demonstrate for the first time that the immunoregulatory action of endocytosed surfactant lipids is mediated at least in part by GSK3 activation. On the other hand, SP-C specifically upregulated basal IL-4 expression, as well as LPS-stimulated cluster of differentiation (CD)200 receptor 3 (CD200R3) mRNA synthesis and inducible nitric oxide synthase (iNOS) production. IL-4 and CD200R3 are involved in the development of alternative immune responses, whereas iNOS is considered to be a pro-inflammatory marker. Altogether, our results provide evidence that both human recombinant SP-C and the lipid component present in synthetic surfactant membranes exert different intracellular immunomodulatory actions once internalized by MH-S cells, balancing the expression of pro- and anti-inflammatory mediators and limiting LPS-induced pro-inflammatory response.

2. INTRODUCTION

The alveolar epithelium is the largest surface of the body in contact with the outside environment. It is continuously exposed to a diverse array of inhaled inorganic particles, allergens and pathogens that escape the defense mechanisms of the upper respiratory airways. To remain healthy, the lungs must have an immune response able to kill and clear pathogens without triggering an excessive inflammatory response, which would damage

alveolar tissues, compromising lung homeostasis and gas-exchange. AMs account for 95% of airspace leukocytes, and are considered to be the main sentinel phagocytic cells of the innate immune system in the lungs (Martin and Frevert 2005; Holt et al. 2008).

In the alveolus, AMs are continuously exposed to pulmonary surfactant, a complex network of extracellular membranes synthesized and secreted into the alveolar space by alveolar type II epithelial cells. Surfactant consists of approximately 90% of lipids (mainly phospholipids) and contains four associated proteins (surfactant proteins A, B, C and D (SP-A, SP-B, SP-C and SP-D)) (Agassandian and Mallampalli 2013). The main function of surfactant is to reduce surface tension at the air-liquid interface in order to prevent the lungs from collapsing at the end of expiration. Surfactant deficiency in immature lungs is the main cause of neonatal respiratory distress syndrome (RDS) (Whitsett and Weaver 2002).

Surfactant components have been recognized to play an essential role in the immune host defense of the lungs (Chroneos et al. 2010; Glasser and Mallampalli 2012). Many of these studies have focused on extracellular actions carried out by hydrophilic SP-A and SP-D, also known as pulmonary collectins (reviewed in Ariki et al. 2012), hydrophobic SP-C (Augusto et al 2003a; Augusto et al. 2003b) and anionic phospholipids, specially palmitoyloleoylphosphatidylglycerol (POPG) (Kuronuma et al. 2009; Numata et al. 2010; Kandasamy et al. 2011; Numata et al. 2012a). However, in the alveolar space surfactant is endocytosed by AMs and type II pneumocytes in order to assure its degradation and recycling (Agassandian and Mallampalli 2013). The effect of these internalized surfactant components on the phenotype or the response of AMs remains largely unknown.

Firstly, SP-A clathrin-dependent endocytosis was reported to activate a protein kinase C (PKC) ζ isoform-dependent pathway that suppressed LPS-induced inflammation in AMs (Moulakakis and Stamme 2009). Secondly, dipalmitoylphosphatidylcholine (DPPC), the most abundant phospholipid molecular species in surfactant monolayers (Bernhard et al. 2001), has been demonstrated to inhibit LPS-elicited IL-8 production in A549 lung epithelial cells by blocking Toll-like receptor 4 (TLR4) translocation into lipid raft domains (Abate et al. 2010). Moreover, DPPC has been proved to modulate the production of platelet-activating factor after LPS stimulation by inhibiting two enzymes involved in its biosynthetic pathway (Tonks et al. 2003), to attenuate LPS-induced respiratory burst via downregulation of PKCδ isoform (Tonks et al. 2005) and to increase cyclooxygenase 2

(COX2) expression and prostaglandin E2 (PGE2) production via phosphorylation of cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) transcription factor in a human monocytic cell line (Morris et al. 2008). In another report, DPPC was shown to regulate the expression of different surface markers in human macrophages (Gille et al. 2007). Interestingly, in these studies DPPC immunoregulatory properties appeared to be dependent on its uptake by the cells. Nevertheless, the mechanism of action of internalized surfactant lipids and proteins in the regulation of the immune response is not fully understood.

In the first chapter of this thesis, a synthetic pulmonary surfactant based on recombinant human SP-C that had previously been shown to be effective in an animal model of lung injury (Ikegami and Jobe 2002) and treating acute RDS patients (Spragg et al. 2003; Spragg et al. 2004), was proved to inhibit LPS-induced response in MH-S mouse AMs. This effect was mediated by POPG in the extracellular medium, and decreased as surfactant vesicles were endocytosed by the cells through a clathrin-dependent mechanism. Since this synthetic surfactant is composed of DPPC, POPG, palmitic acid (PA) and recombinant human SP-C, it seems possible that its components may promote different immunoregulatory actions after being internalized by the cells.

Even though any report has been published about the intracellular action of SP-C, this protein seems to play an important role in the regulation of the activation state of AMs. Mutations in the gene encoding SP-C cause interstitial lung disease in humans, a disease characterized by structural remodeling of the distal spaces and impaired gas-exchange, often leading to the development of fibrosis in children and adults (Glasser et al. 2010; Gower and Nogee 2011). Furthermore, SP-C-deficient mice were shown to develop pneumonitis and emphysema (Glasser et al. 2003), increased and prolonged fibrosis after bleomycin exposure (Lawson et al. 2005) and susceptibility to *Pseudomonas aeruginosa* (Glasser et al. 2008) and respiratory syncytial virus infections (Glasser et al. 2009; Glasser et al. 2013a), all these linked to dysregulated immune responses and altered activity and phenotype of AMs.

Taking everything into consideration, the purpose of this study was to evaluate the intracellular immunomodulatory action of synthetic surfactant vesicles internalized by AMs on the activation state of these cells after being stimulated with LPS. More specifically, the

aims were to determine which of its components exerted this effect and which signaling pathways were involved in it.

3. EXPERIMENTAL DESIGN

As we have already mentioned in chapter 1, the synthetic surfactant based on recombinant human SP-C contains 98% of lipids by weight (DPPC/POPG/PA 2.3:1:0.16 w/w) and 2% of human recombinant SP-C. SP-C is a small hydrophobic peptide with a α-helix segment which adopts a transmembrane orientation, and a more polar N-terminal segment with two palmitoylated cysteines (Vandenbussche et al. 1992a). However, recombinant SP-C is not palmitoylated (see chapter 1 section 3). Surfactant small unilamellar or multilamellar vesicles (SUVs or MLVs) were prepared as described in materials and methods.

To evaluate the immunomodulatory action of internalized surfactant vesicles on the activation state of MH-S murine AMs, these cells were preincubated with synthetic surfactant membranes containing human recombinant SP-C (sSPC), or surfactant membranes without SP-C (sPL), for 18 or 24 hours (figure 1). This preincubation time was long enough to allow surfactant endocytosis, as proved by confocal microscopy studies.

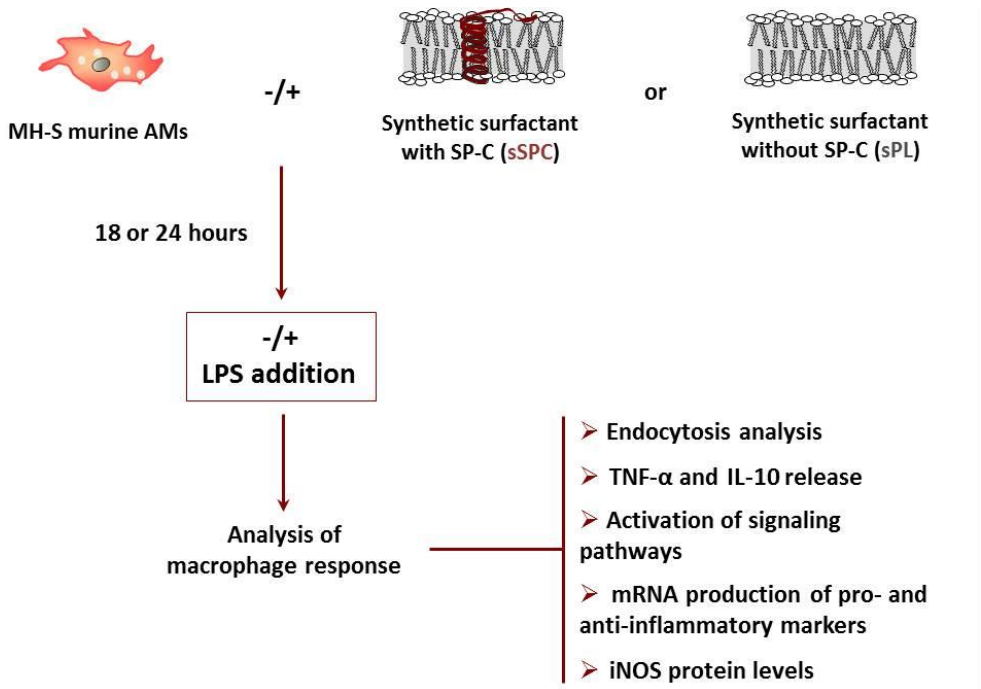


Figure 1: Experimental design used in chapter 2.

After that, macrophages were stimulated or not with smooth bacterial LPS from *E. coli*, and their activation state was analyzed by different methods. TNF-α and IL-10 releases were measured by enzyme-linked immune sorbent assay (ELISA); mRNA production of different pro- and anti-inflammatory markers was quantified by quantitative real-time polymerase chain reaction (qPCR); and activation of signaling pathways and iNOS protein levels were analyzed by western-blot.

In some experiments, lithium chloride was added to the cells for one hour after the preincubation with surfactant vesicles and before the stimulation with LPS, in order to inhibit GSK3 activity. Then, TNF-α and IL-10 releases were measured by ELISA and the expression of different immune related genes was determined by qPCR.

4. RESULTS

4.1 Surfactant vesicle endocytosis

In the alveolar space, surfactant is endocytosed by AMs in order to assure its degradation (Agassandian and Mallampalli 2013). In the first chapter of this thesis, we proved that synthetic surfactant SUVs were endocytosed by MH-S cells by a clathrin-dependent mechanism. This process began almost ten minutes after the addition of the vesicles, and increased as the incubation time augmented. In this work, initial confocal microscopy experiments were conducted to determine if synthetic surfactant MLVs were also internalized by MH-S AMs. For this purpose, the cells were incubated with DiI-labeled sPL or sSPC SUVs or MLVs for 24 hours. Then, macrophages were fixed, nuclei were stained with DAPI, and CD14 (a protein present in the surface of AMs which binds to LPS (Wright et al. 1990; Hailman et al. 1994)) was detected with an FITC-conjugated antibody. As it is shown in confocal micrographs (figure 2), after a 24 hour-long incubation, sPL and sSPC SUVs had been endocytosed by the cells whereas synthetic surfactant MLVs were not observed inside MH-S AMs.

Furthermore, surfactant vesicle endocytosis was also analyzed by flow cytometry after incubating MH-S cells for 24 hours with DiI-labeled sPL or sSPC SUVs or MLVs. As we can see in the histogram plots (figure 3), cells incubated with sPL or sSPC SUVs presented higher fluorescence intensity than cells incubated with synthetic surfactant MLVs. This

result indicates that sPL and sSPC SUVs are endocytosed to a higher extent than surfactant MLVs, accordingly with confocal microscopy observations.

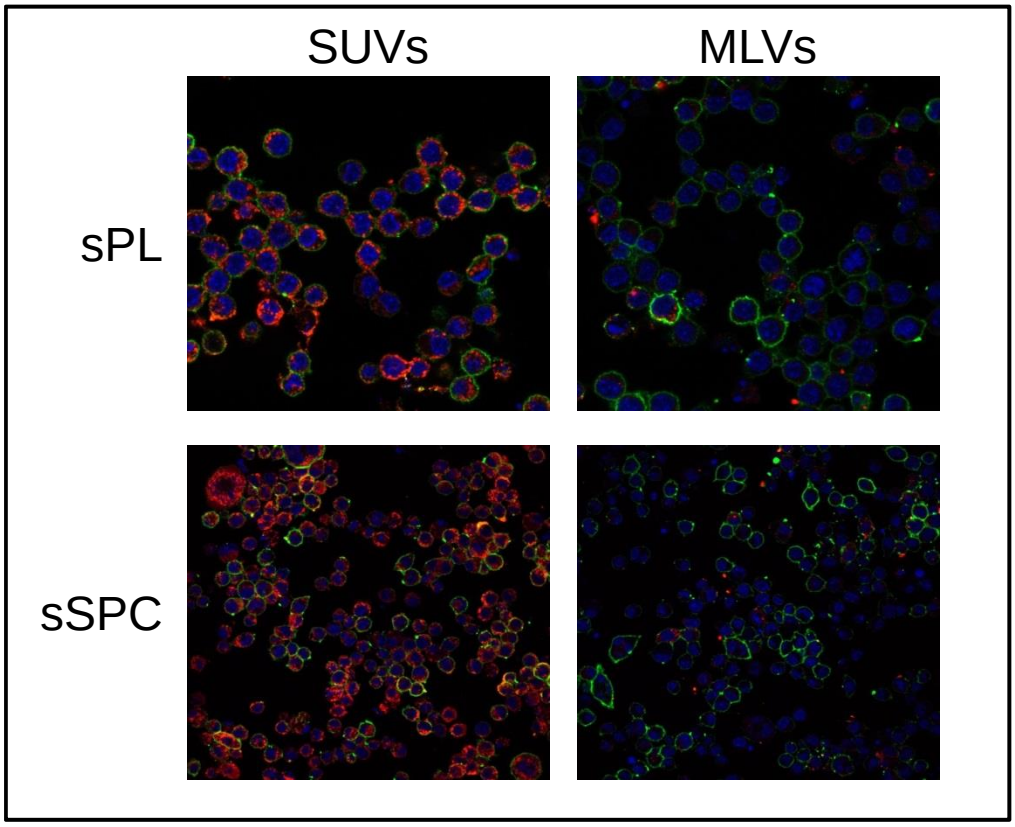


Figure 2: sPL and sSPC MLVs are endocytosed to a lesser extent than surfactant SUVs by MH-S cells. Macrophages were incubated with 250 μg/ml DiI-labeled sPL or sSPC vesicles (in a 200:1 phospholipids:probe molar ratio) (in red) for 24 hours. Then cells were fixed, nuclei were stained with 1 μg/ml DAPI (in blue) and CD14 was detected with an FITC-conjugated antibody (in green). Confocal micrographs are representative of three independent experiments.

Taking these findings into consideration, only synthetic surfactant SUVs were used in the following experiments, in order to guarantee their endocytosis by MH-S murine AMs.

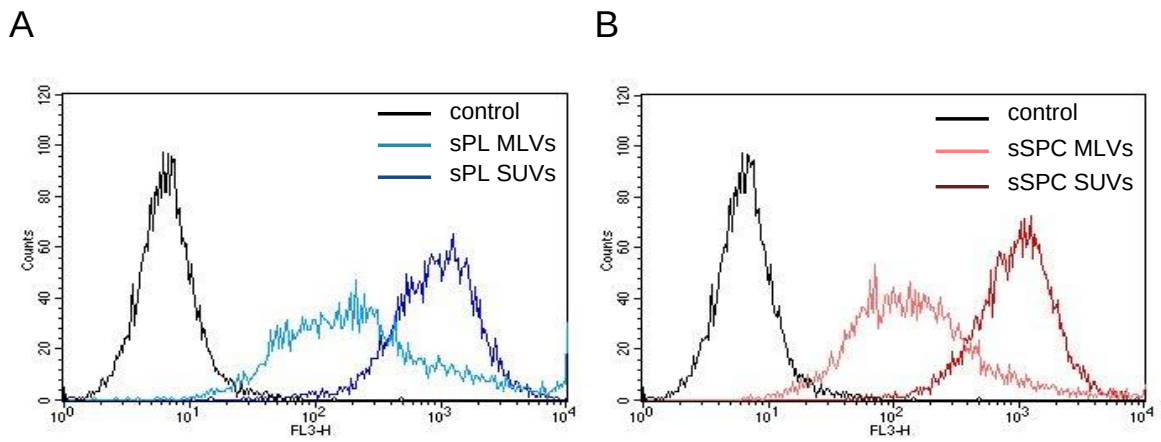


Figure 3: sPL and sSPC SUVs are endocytosed to a higher extent than surfactant MLVs. MH-S cells were incubated with 250 μg/ml DiI-labeled sPL or sSPC vesicles (in a 200:1 phospholipids:probe molar ratio) for 24 hours. Cells were then harvested and analyzed by flow cytometry. Histogram plots from a representative experiment are depicted in (A) and (B).

4.2 Inhibition of LPS-induced TNF-α production

TNF-α is a pro-inflammatory cytokine commonly produced by LPS-challenged macrophages (Bode et al. 2012). To examine if internalized synthetic surfactant vesicles were able to modify the activation state of AMs, we first determined TNF-α mRNA production and release by MH-S cells preincubated with sPL or sSPC SUVs for 18 hours and subsequently stimulated or not with bacterial LPS. As it is shown in figure 4, basal TNF-α expression and release were almost undetectable, and were not affected by the preincubation with sPL or sSPC. However, surfactant SUVs significantly attenuated LPS-elicited TNF-α mRNA production (figure 4A) and secretion (figure 4B). This inhibition was similar in the presence or absence of SP-C. Therefore, we concluded that the modulatory effect on LPS-induced TNF-α expression and release is carried out by the lipid component of synthetic surfactant. Interestingly, the decrease in TNF-α secretion (figure 4B) was much more pronounced than the attenuation of its expression (figure 4A). This suggests that internalized synthetic surfactant lipids must be regulating additional posttranscriptional steps, leading to a lower release of TNF-α to the extracellular medium.

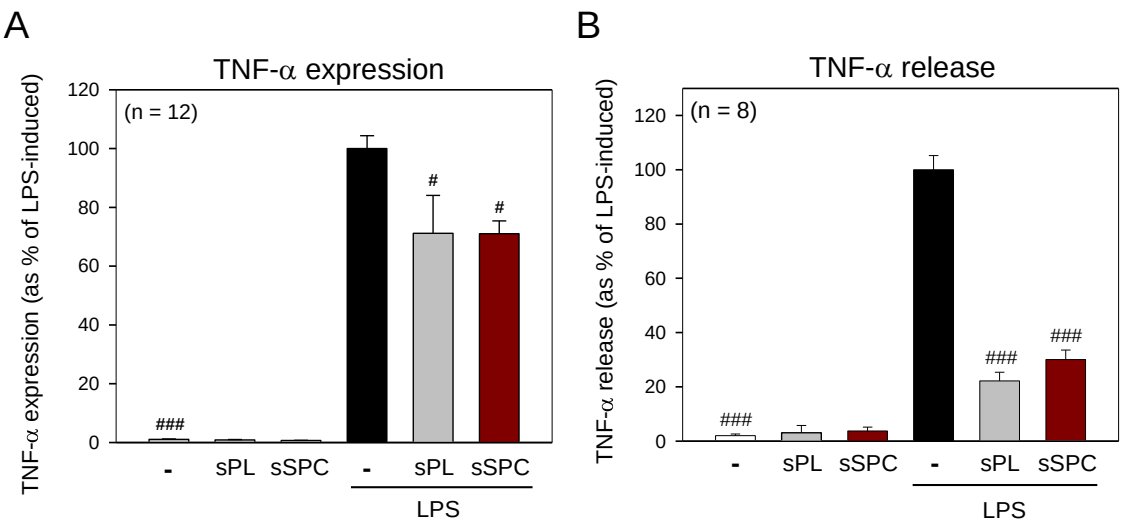


Figure 4: sPL and sSPC SUVs inhibit LPS-induced TNF-α mRNA production and release. MH-S cells were preincubated with 250 μg/ml sPL or sSPC SUVs for 18 hours and stimulated with LPS (1 μg/ml) for 1 (A) or 4 (B) hours. (A) TNF-α mRNA production was measured by qPCR. Data are expressed as percentages of LPS-induced TNF-α expression ± standard error (SE). (B) TNF-α release was measured by ELISA. Data are expressed as percentages of LPS-induced TNF-α release ± SE. Basal TNF-α secretion was 4 ± 1 pg/ml, and the average LPS-induced TNF-α release in the absence of surfactant was 697.7 ± 117 pg/ml. (#$p<0.05$, ###$p<0.001$ vs LPS).

Next, we analyzed if sPL or sSPC MLVs had the same inhibitory properties on LPS-induced TNF-α secretion as synthetic surfactant SUVs. As we can see in figure 5, only surfactant SUVs were able to decrease LPS-elicited TNF-α release, whereas sPL and sSPC MLVs failed to show any modulatory effect. This is probably due to the fact that synthetic surfactant MLVs cannot be endocytosed by the cells (figure 2) and remain in the extracellular medium. Therefore, after an 18 hour-long incubation, vesicles that have not been internalized by MH-S macrophages are not able to regulate LPS-induced TNF-α production.

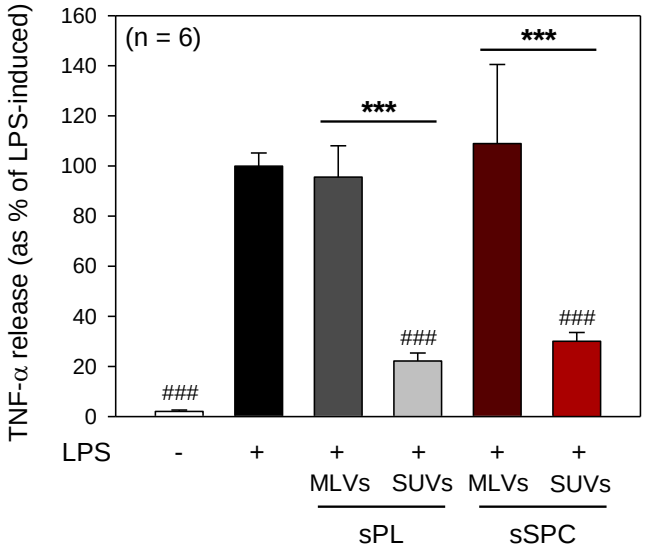


Figure 5: Unilamellar but not multilamellar surfactant vesicles inhibit LPS-induced TNF-α release. MH-S cells were preincubated with sPL or sSPC SUVs or MLVs (250 μg/ml) for 18 hours and stimulated with LPS (1 μg/ml) for 4 hours. TNF-α secretion was measured by ELISA. Data are expressed as percentages of LPS-induced TNF-α release ± SE. Basal TNF-α secretion was 4 ± 1 pg/ml, and the average LPS-induced TNF-α release in the absence of surfactant was 697.7 ± 117 pg/ml (***$p<0.001$ between indicated groups; ###$p<0.001$ vs LPS).

4.3 Inhibition of IL-10 release

The following step consisted in studying if internalized surfactant vesicles were able to modify the release of IL-10, which is an anti-inflammatory cytokine produced also by LPS-stimulated macrophages (Bode et al. 2012). MH-S cells were preincubated with sPL or sSPC SUVs for 18 hours and subsequently stimulated or not with bacterial LPS. As it is shown in figure 6, MH-S AMs presented a high basal IL-10 release, which was modestly increased in the presence of LPS, although a higher concentration of endotoxin (2 μg/ml) was required to detect this increment. Preincubation of the cells with sPL or sSPC SUVs attenuated both basal and LPS-induced production of IL-10. This inhibition was similar in the absence or presence of SP-C, which allowed us to conclude that synthetic surfactant lipid component was also responsible for the modulation of basal and LPS-elicited IL-10 secretion. Furthermore, these results demonstrate that internalized surfactant lipids modify the activation state of AMs even in the absence of a pro-inflammatory stimulus.

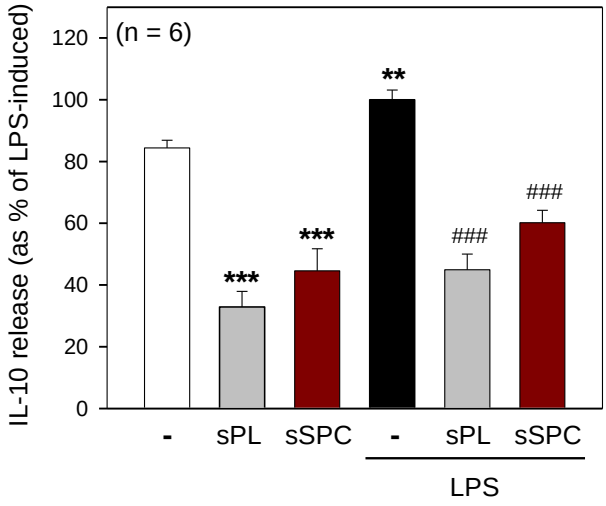


Figure 6: sPL and sSPC SUVs inhibit IL-10 release in the absence or presence of LPS. MH-S cells were preincubated with 250 μg/ml sPL or sSPC SUVs for 18 hours and stimulated with LPS (2 μg/ml) for 4 hours. IL-10 secretion was measured by ELISA. Data are expressed as percentages of LPS-induced IL-10 release ± SE. Basal IL-10 secretion was 115 ± 3 pg/ml, and the average LPS-induced IL-10 release in the absence of surfactant was 137 ± 4 pg/ml. (**p<0.01, ***p<0.001 vs control; ###p<0.001 vs LPS).

4.4 Inhibition of signaling pathways

In the alveolar space, LPS molecules bind to LPS-binding protein (LBP) present in the alveolar fluid. LBP transfers LPS to CD14, which can be found in a soluble form or anchored to AM cell membrane (Wright et al. 1990; Hailman et al. 1994). Then, CD14 presents LPS to its receptor complex on the surface of AMs, composed of TLR4 and MD-2 (Poltorak et al. 1998; Nagai et al. 2002). The interaction of LPS with the TLR4/MD2 complex triggers the oligomerization of TLR4 and subsequent activation of different signaling cascades. Among those are the pathways involving PI3K and Akt (O'Toole and Peppelenbosch 2007); NF-κB transcription factor; and extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPKs. This leads to production of pro-inflammatory mediators such as TNF-α and anti-inflammatory cytokines such as IL-10 (reviewed in Bode et al. 2012).

The purpose of our next experiments was to investigate the effect of internalized synthetic surfactant on the activation of these signaling pathways, in the absence or presence of LPS. MH-S AMs were preincubated with surfactant SUVs for 18 hours and afterwards stimulated or not with LPS. We first examined the phosphorylation and activation of ERK and p38 MAPKs (figure 7).

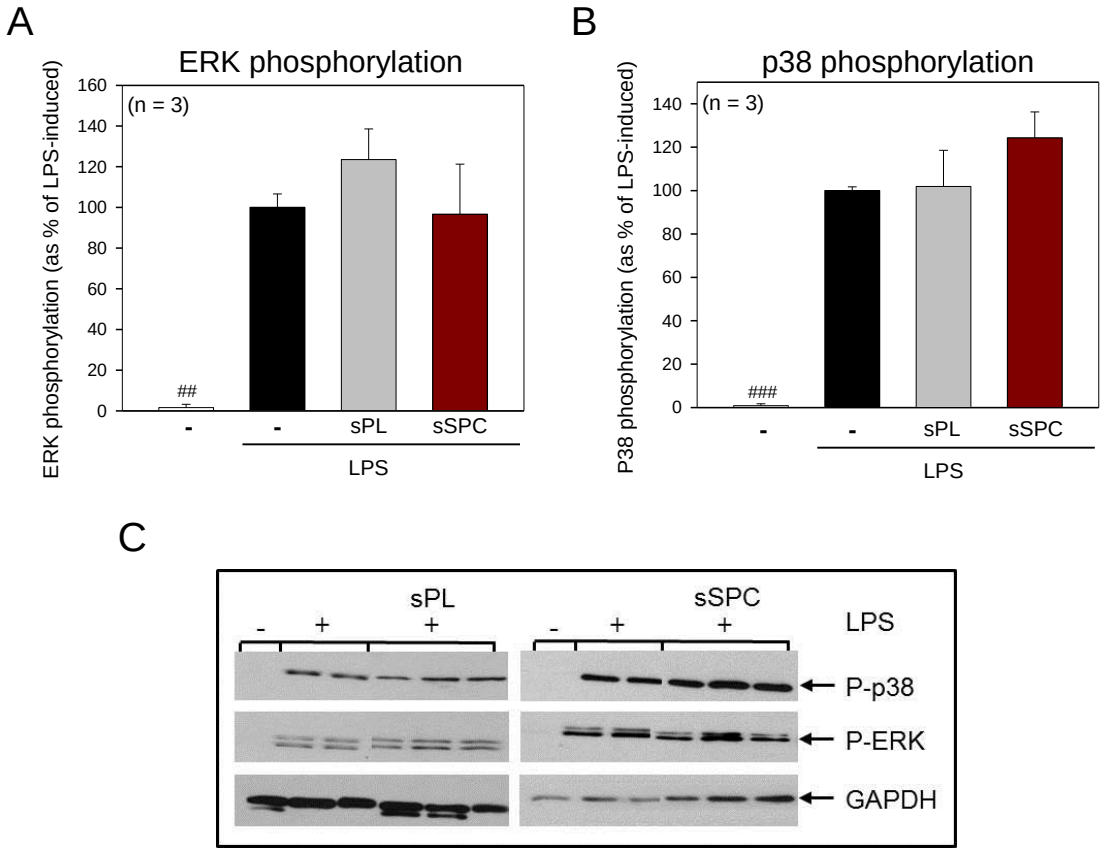


Figure 7: Surfactant SUVs do not inhibit LPS-induced ERK and p38 phosphorylations. MH-S cells were preincubated with 250 μg/ml sPL or sSPC SUVs for 18 hours and stimulated with LPS (1 μg/ml) for 10 minutes. Then, ERK (A) and p38 (B) phosphorylations were detected by western-blot with phospho-specific antibodies, and quantified by densitometry using GAPDH as loading control. LPS-induced phosphorylation level in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (C) Representative western-blot images of MH-S cells treated as explained above. (##p<0.01, ###p<0.001 vs LPS).

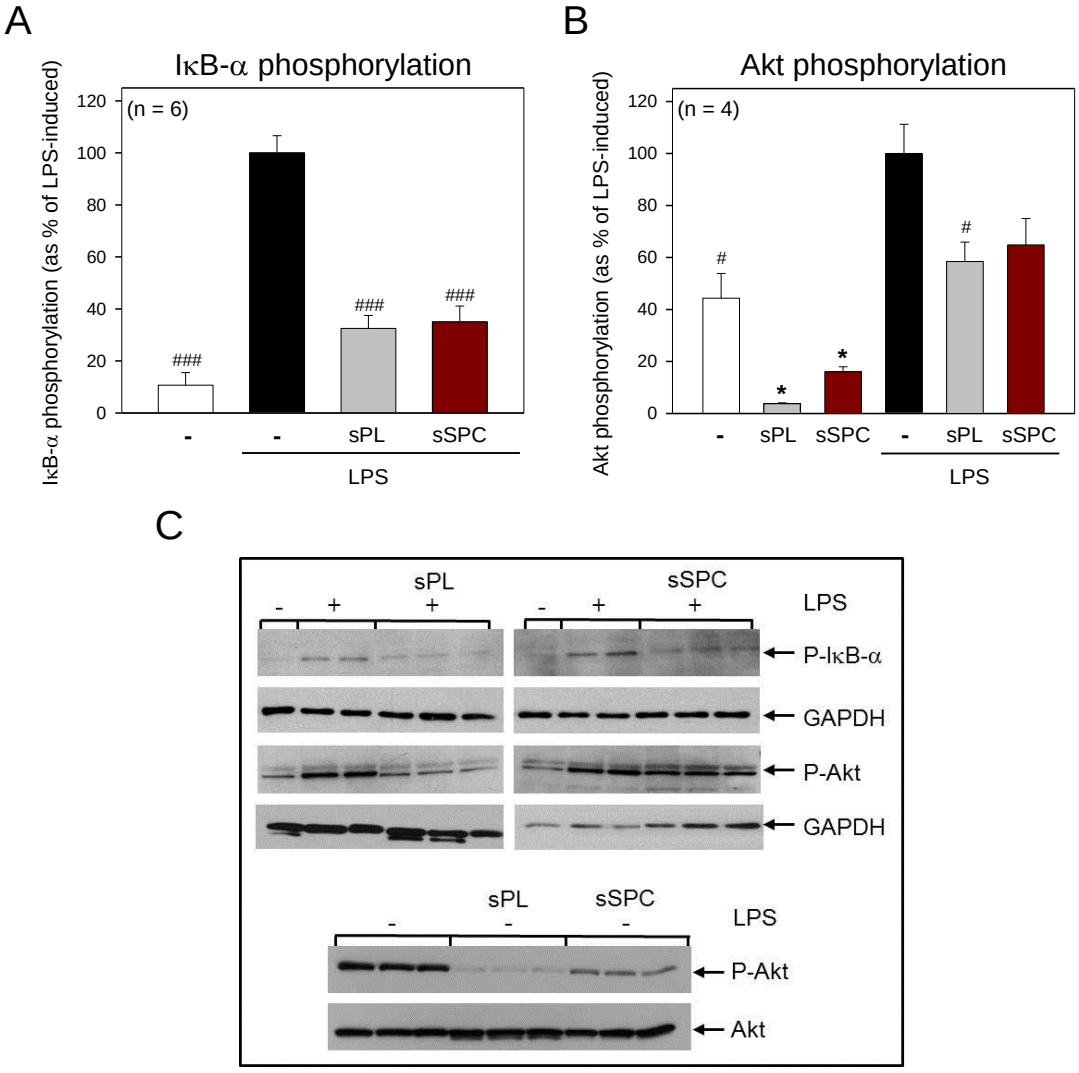


Figure 8: Surfactant SUVs inhibit IκB-α and Akt phosphorylations. MH-S cells were preincubated with 250 μg/ml sPL or sSPC SUVs for 18 hours and stimulated with LPS (1 μg/ml) for 30 (A) or 10 (B) minutes. Then, IκB-α (A) and Akt (B) phosphorylations were detected by western-blot with phospho-specific antibodies, and quantified by densitometry using GAPDH or total protein as loading controls. LPS-induced phosphorylation level in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (C) Representative western-blot images of MH-S cells treated as explained above. (*$p<0.05$ vs control; #$p<0.05$, ###$p<0.001$ vs LPS).

The phosphorylation of ERK and p38 MAPKs was undetectable in the absence of LPS, even after the preincubation with surfactant vesicles (data not shown). However, LPS-

stimulation induced the phosphorylation and activation of both proteins. Preincubation of the cells with sPL and sSPC SUVs did not affect LPS-elicited ERK and p38 phosphorylation levels, indicating that the immunomodulatory effect of internalized synthetic surfactant vesicles is not mediated by the inhibition of these MAPKs.

We next examined the phosphorylation of IκB-α and Akt. IκB-α is NF-κB inhibitor, its phosphorylation promotes its degradation and allows NF-κB activation (Newton and Dixit 2012). Akt is a key PI3K effector protein, phosphorylated and activated as a consequence of PI3K activity (Vanhaesebroeck et al. 2012). As happened with ERK and p38 MAPKs, the phosphorylation of IκB-α was almost unappreciable in the absence of LPS independently of the preincubation with surfactant vesicles (data not shown), but increased after LPS stimulation (figure 8A). However, the preincubation with sPL and sSPC SUVs decreased LPS-elicited IκB-α phosphorylation, and therefore, promoted the inactivation of NF-κB. On the other hand, Akt phosphorylation was detected even in the absence of endotoxin, although it augmented after LPS stimulation (figure 8B). Synthetic surfactant vesicles diminished significantly both basal and LPS-induced Akt phosphorylation. This modulatory effect on Akt and NF-κB activation was also carried out by the lipid component of synthetic surfactant, since the inhibition was similar for sPL and sSPC vesicles.

The fact that internalized synthetic surfactant vesicles down-regulated the activation of PI3K/Akt and NF-κB pathways but showed no modulatory effect on the phosphorylation of ERK and p38 MAPKs allowed us to conclude that the immunoregulatory action of endocytosed surfactant lipids must take place downstream of the TLR4/MD2 receptor complex.

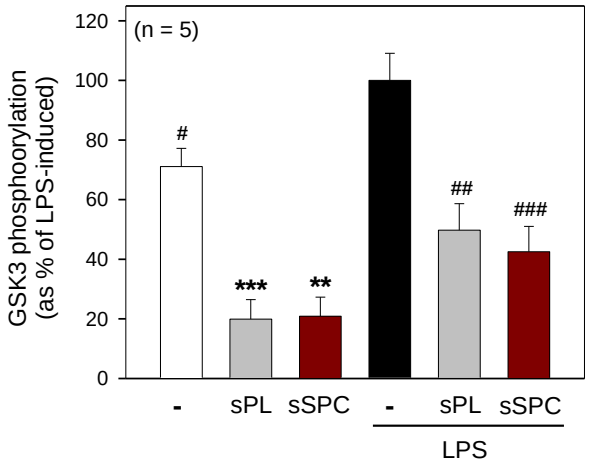
4.5 GSK3 activation by surfactant preincubation

One of the downstream targets of PI3K is GSK3, which is a constitutively active serine/threonine kinase with two isoforms (GSK3-α and GSK3-β). It can be inactivated through phosphorylation by several protein kinases, including Akt. GSK3 is involved in the regulation of a multitude of physiological processes through its ability to regulate a variety of signaling proteins and transcription factors, including NF-κB, CREB, signal transducer and activator of transcription 3 and 5 (STAT3, STAT5) and nuclear factor of activated T

cells (NF-AT), among others. For this reason, GSK3 is considered to play a pivotal role in the regulation of the expression of pro- and anti-inflammatory cytokines (Hazeki et al. 2007; Wang et al. 2011).

Considering that internalized synthetic surfactant lipids inhibited basal and LPS-induced Akt phosphorylation and activation (figure 8B), our next step was to analyze if the preincubation of MH-S AMs with sPL or sSPC SUVs for 18 hours had a regulatory effect on GSK3 activity in the presence or absence of LPS.

A



B

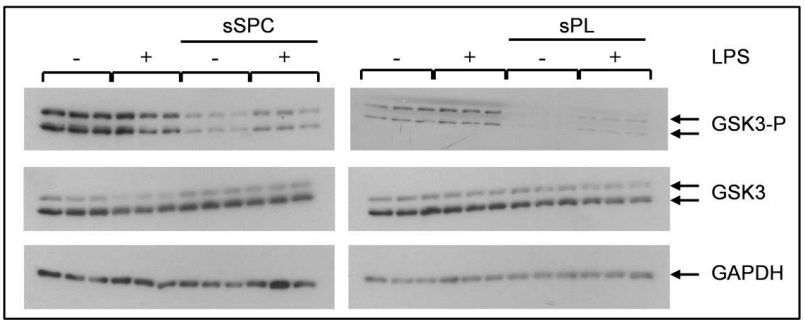


Figure 9: sPL and sSPC induce GSK3 activation in the absence or presence of LPS. (A) MH-S cells were preincubated with 250 µg/ml sPL or sSPC SUVs for 18 hours and stimulated with LPS (1 µg/ml) for 20 minutes. GSK3α/β phosphorylation levels were detected by western-blot and quantified by densitometry. LPS-induced GSK3 phosphorylation in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (**p<0.01, ***p<0.001 vs control; #p<0.05, ##p<0.01, ###p<0.001 vs LPS). (B) Representative GSK3 western-blot images of MH-S cells treated as described in A.

As we can see in figure 9, both isoforms of GSK3 were phosphorylated under basal conditions, although their phosphorylation level increased modestly after LPS stimulation, as happened with Akt phosphorylation (figure 8B). The preincubation with sPL and sSPC SUVs decreased both basal and LPS-induced GSK3 phosphorylation, resulting in GSK3 activation. This effect was similar in the absence or presence of SP-C, indicating that synthetic surfactant lipid component is also responsible for the activation of GSK3. Therefore, these findings suggested that internalized synthetic surfactant lipids decrease basal and LPS-elicited Akt activation, which results in increased GSK3 activity in the absence or presence of LPS.

4.6 Increased expression of inflammatory markers

So far, we have demonstrated that internalized synthetic surfactant lipids were able to decrease basal and LPS-elicited IL-10 release (figure 6) and Akt activation (figure 8B), and that they promoted GSK3 activation in the absence or presence of LPS (figure 9). Moreover, they also inhibited LPS-induced NF-κB activation (figure 8A) and TNF-α production (figure 4). Furthermore, this effect is likely to take place downstream of the LPS TLR4/MD2 receptor complex, because the preincubation with sPL SUVs did not modulate the activation of ERK nor p38 MAPKs (figure 7).

In order to examine whether internalized synthetic surfactant lipids were also able to modify the expression of other inflammatory markers, we analyzed by qPCR the expression of interferon regulatory factor 1 (IRF1), COX2, C-X-C motif chemokines 10 and 11 (CXCL10 and CXCL11), CD80 and suppressor of cytokine signaling 3 (SOCS 3) in MH-S AMs preincubated with sPL or sSPC SUVs for 18 hours and subsequently stimulated or not with bacterial LPS.

These proteins have been shown to play a role in the development of immune responses. IRF1 stimulates the expression of interferon-responsive genes and pro-inflammatory cytokines and promotes Th1 responses (Ozato et al. 2007). COX2 is the key enzyme regulating the production of prostaglandins, which are central mediators of inflammation (Tsatsanis et al. 2006). CXCL10 and CXCL11 are pro-inflammatory cytokines known to induce chemotaxis in different cells of the immune system, including activated Th1 cells and NK cells (Widney et al. 2000; Liu et al. 2011). CD80 is a

costimulatory molecule expressed by antigen-presenting cells, necessary for T cell activation (Lim et al. 2005). Finally, SOCS 3 prevents cytokines such as IL-6 from untimely mediating IL-10-like suppressive effects via induction of a sustained STAT 3 activation (Bode et al. 2012). Previous preliminary studies in our laboratory had shown that the expression of these proteins was induced after LPS stimulation in MH-S cells (data not shown).

As we can see in figure 10, stimulation of MH-S AMs with LPS elicited an increase in the expression of these proteins. Basal mRNA production was not affected by the preincubation with synthetic surfactant vesicles. However, the preincubation with sPL or sSPC SUVs augmented significantly the expression of the six proteins after the stimulation with LPS. This surprising effect was similar in the absence or presence of SP-C, confirming that internalized surfactant lipids were responsible for the up-regulation of the expression of these genes in the presence of LPS.

The effect of internalized synthetic surfactant lipids on IRF1, COX2, CXCL10, CXCL11, CD80 and SOCS 3 expressions was opposite to the one we had observed with TNF-α and IL-10 productions (figures 4 and 6). This led us to suggest that the signaling pathways and transcription factors involved in the regulation of the expression of IRF1, COX2, CXCL10, CXCL11, CD80 and SOCS 3 must differ from the ones modulating TNF-α and IL-10 transcription, at least in MH-S AMs.

Stimulation of macrophages with LPS triggers also the activation of IRF3 transcription factor, which binds to interferon-stimulated response element (ISRE) and together with NF-κB activates the transcription of target genes such as type I interferons and some interferon-inducible genes (Lu et al. 2008; Bode et al. 2012). For this reason, we wanted to analyze the effect of the preincubation with surfactant vesicles on the expression of type I interferons alpha and beta (IFN-α and INF-β), and type II interferon gamma (IFN-γ) in the presence or absence of LPS. However, no basal or LPS-induced expression of these genes was found in MH-S AMs (data not shown).

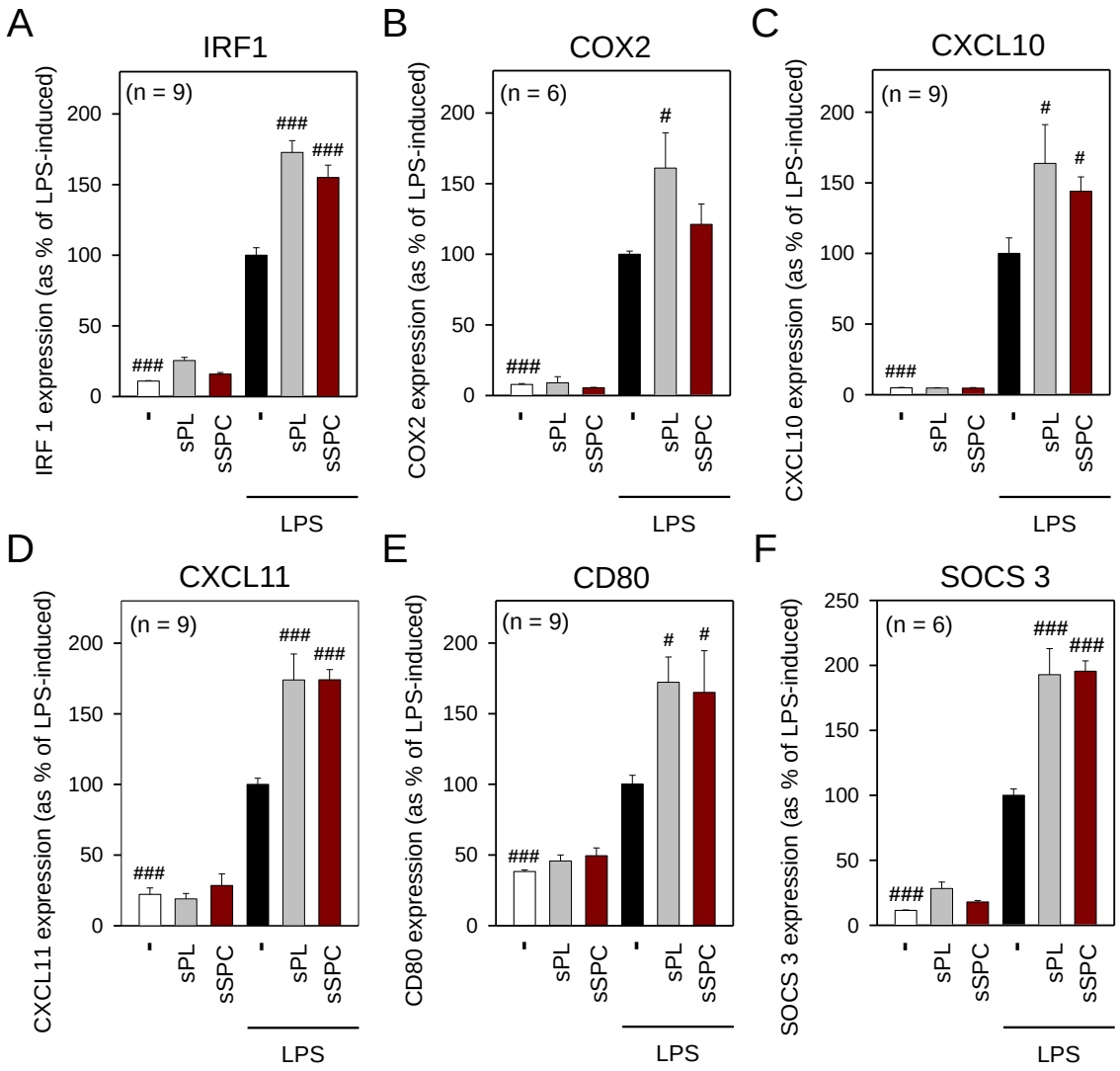


Figure 10: sPL and sSPC increase LPS-induced IRF1 (A), COX2 (B), CXCL10 (C), CXCL11 (D) CD80 (E), and SOCS 3 (F) expressions. MH-S cells were preincubated with 250 μg/ml sPL or sSPC SUVs for 18 hours and stimulated with LPS (1 μg/ml) for 1 hour. RNA was then extracted for qPCR. LPS-induced expression in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (#p<0.05, ###p<0.001 vs LPS).

4.7 Effect of GSK3 inhibition on sPL-induced immunomodulatory action

Internalized synthetic surfactant lipids have been shown to inhibit Akt activation and to activate GSK3 both in the absence or presence of LPS (figures 8B and 9). As GSK3 has been reported to play an essential role in the regulation of the expression of pro- and anti-inflammatory cytokines (Hazeki et al. 2007; Wang et al. 2011), our next step consisted in studying if sPL-induced modulation of the expression and production of inflammatory markers is mediated by the activation of GSK3.

For that purpose, MH-S AMs were preincubated with sPL SUVs for 18 hours. Then, 10 mM LiCl was added to the cells for one hour. Lithium is a direct and indirect inhibitor of GSK3: it acts as a competitive inhibitor of Mg^{2+} (required for GSK3 activity), and increases the phosphorylation and inactivation of both GSK3 isoforms (Jope 2003). After that, macrophages were stimulated or not with LPS and TNF-α and IL-10 releases were determined by ELISA.

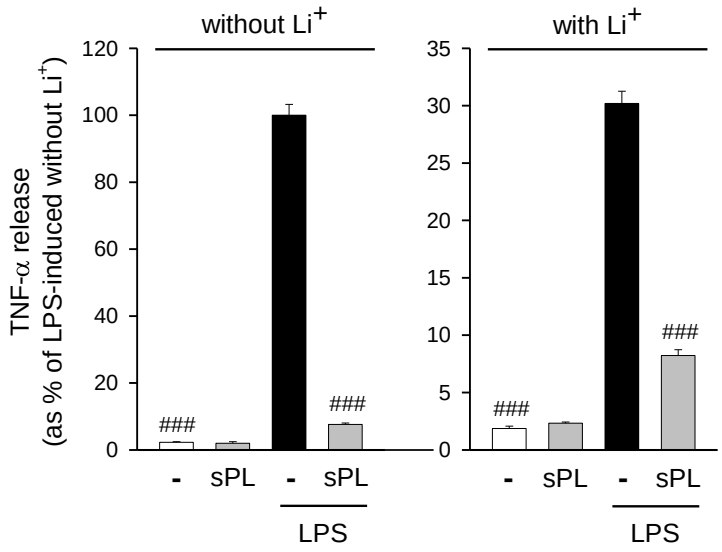


Figure 11: Lithium-induced GSK3 inhibition reduces sPL immunomodulatory effect on LPS-elicited TNF-α release. MH-S cells were preincubated with 250 μg/ml sPL SUVs for 18 hours. Then 10 mM LiCl was added to the cells for 1 hour, prior to the stimulation with LPS (1 μg/ml) for 4 hours. TNF-α secretion was measured by ELISA. Data are expressed as percentages of LPS-induced TNF-α release in the absence of lithium and sPL ± SE. Basal TNF-α secretion was 9 ± 1 pg/ml, and the average LPS-induced TNF-α release in the absence of surfactant and lithium was 397 ± 13 pg/ml. (###$p<0.001$ vs LPS).

Firstly, the results concerning TNF-α release are depicted in figure 11. In the presence of lithium, LPS-induced TNF-α release decreased to 30 ± 1 % of the one obtained in the absence of lithium. Nevertheless, sPL SUVs attenuated LPS-elicited TNF-α secretion by 92.4 ± 0.4 % in the absence of lithium, whereas this down-regulation decreased to 72.8 ± 1.7 % after GSK3 inhibition. These inhibition percentages are significantly different ($p<0.001$). This finding indicates that sPL immunomodulatory action on LPS-induced TNF-α release is mediated at least in part by GSK3 activation.

Secondly, IL-10 release is shown in figure 12. As happened with TNF-α, in the presence of lithium, LPS-induced IL-10 secretion decreased to 20 ± 2 % of the one obtained in the absence of lithium. Accordingly with the results obtained in figure 6, sPL SUVs reduced basal and LPS-elicited IL-10 release in the absence of lithium. However, this modulatory effect was abrogated after GSK3 inhibition. This finding allowed us to conclude that sPL-induced regulation of IL-10 release is also mediated by GSK3 activation.

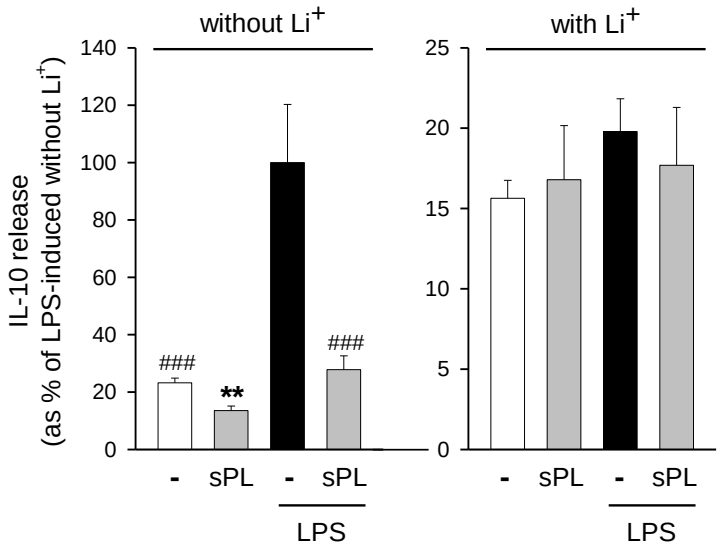


Figure 12: Lithium-induced GSK3 inhibition suppresses sPL immunomodulatory effect on IL-10 release. MH-S cells were preincubated with 250 μg/ml sPL SUVs for 18 hours. Then 10 mM LiCl was added to the cells for 1 hour, prior to the stimulation with LPS (2 μg/ml) for 4 hours. IL-10 secretion was measured by ELISA. Data are expressed as percentages of LPS-induced IL-10 release in the absence of lithium and sPL ± SE. Basal IL-10 secretion was 19 ± 1 pg/ml, and the average LPS-induced IL-10 release in the absence of surfactant and lithium was 93 ± 20 pg/ml. (**$p<0.01$ vs control; ###$p<0.001$ vs LPS).

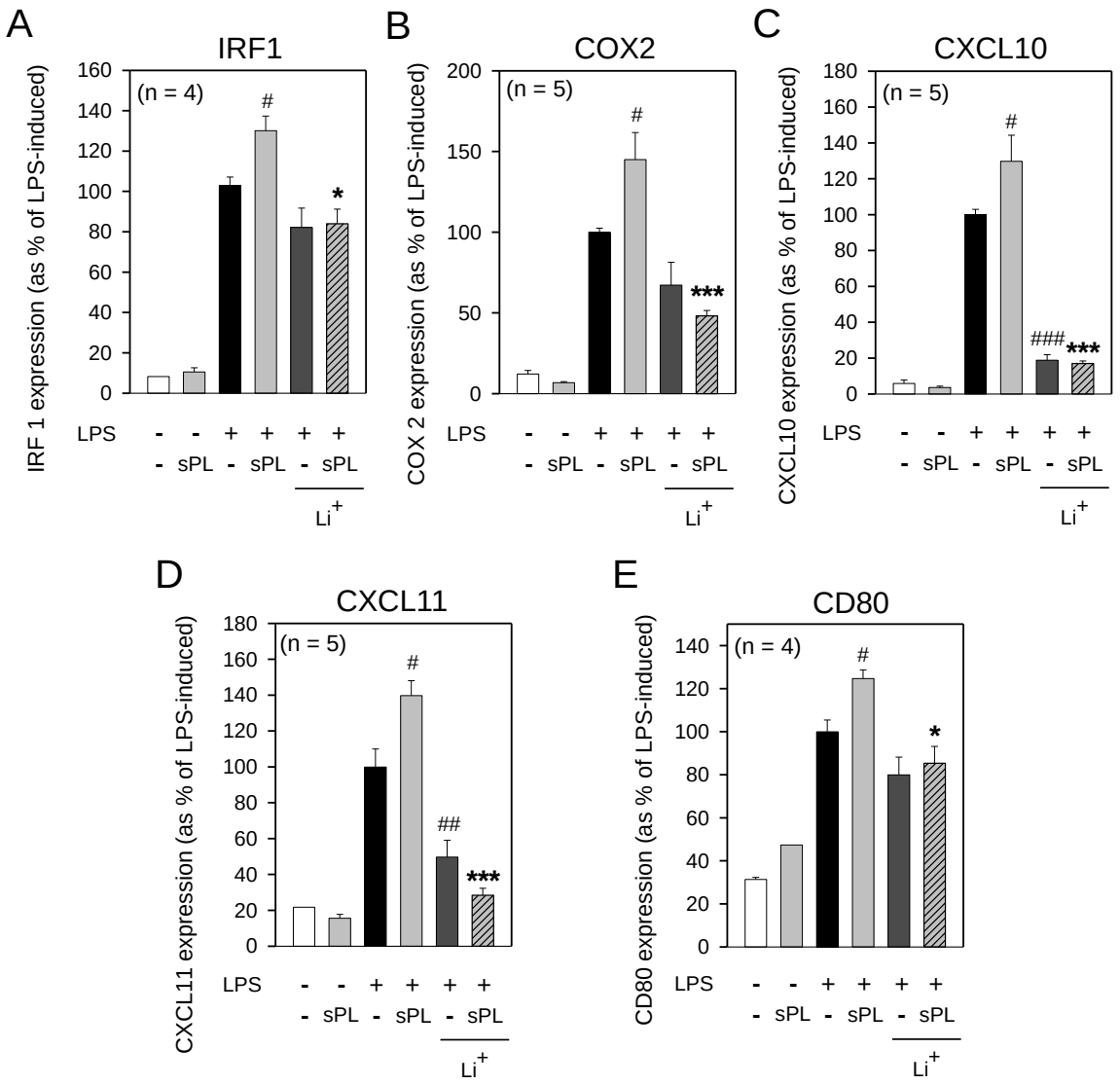


Figure 13: GSK3 inhibition abrogates sPL-induced increase in IRF1 (A), COX2 (B), CXCL10 (C), CXCL11 (D) and CD80 (E) expressions after LPS stimulation. MH-S cells were preincubated with 250 μg/ml sPL SUVs for 18 hours. Then 10 mM LiCl was added to the cells for one hour, prior to the stimulation with LPS (1 μg/ml) for another hour. RNA was then extracted for qPCR. LPS-induced expression in the absence of surfactant and lithium was set at 100%. Data are expressed as mean percentages ± SE. (*p<0.05, ***p<0.001 vs the analogue without lithium; #p<0.05, ##p<0.01, ###p<0.001 vs LPS).

Additionally, our next experiments examined the effect of GSK3 inhibition on sPL-induced up-regulation of the expression of IRF1, COX2, CXCL10, CXCL11 and CD80

after the stimulation with LPS. MH-S AMs were preincubated with sPL SUVs for 18 hours, and then LiCl was added to the cells for one hour prior to the stimulation with LPS. After that, the expression of these inflammatory markers was analyzed by qPCR. As we can see in figure 13, lithium significantly decreased LPS-elicited expression of CXCL10 and CXCL11, although the same tendency was observed for IRF1, COX2, and CD80. Moreover, GSK3 inhibition suppressed sPL-induced increase in the expression of the five proteins. These results allowed us to conclude that the up-regulation of LPS-elicited expression of IRF1, COX2, CXCL10, CXCL11 and CD80 exerted by internalized sPL SUVs is also mediated by GSK3 activation.

Altogether, these results demonstrate that the immunomodulatory action of internalized synthetic surfactant lipids on the expression and production of inflammatory markers is mediated at least in part by the activation of GSK3.

4.8 SP-C-induced increase in the expression of IL-4, CD200R3 and iNOS

Apart from synthetic surfactant lipid component, we hypothesized that recombinant human SP-C present in sSPC membranes could also have immunomodulatory properties once endocytosed by AMs. SP-C is considered to play an important role in the regulation of the activation state of AMs, as proved by studies performed with SP-C-deficient mice (Glasser et al. 2003; Lawson et al. 2005; Glasser et al. 2008; Glasser et al. 2009; Glasser et al. 2013a) and reports concerning individuals with mutations in the gene encoding SP-C (Glasser et al. 2010; Gower and Nogee 2011). Moreover, previous preliminary experiments conducted in our laboratory had shown that the expression of IL-4, CD200R3 and iNOS was up-regulated specifically by sSPC internalized vesicles in MH-S cells. To confirm it, MH-S AMs were preincubated with sPL or sSPC SUVs for 18 hours and stimulated or not with LPS. After that, the expression of the three proteins was determined by qPCR and iNOS protein levels were analyzed by western-blot.

IL-4 is a cytokine that induces an alternative or M2 activation in macrophages. M2 macrophages suppress inflammation and antitumor immunity, facilitate wound repair, regulate glucose metabolism, and are involved in the development of Th2 immune responses against extracellular parasites and fungi (Galli et al. 2011; Murray and Wynn 2011). As it is shown in figure 14A, basal IL-4 expression was increased specifically by

sSPC SUVs. LPS-stimulation failed to induce IL-4 mRNA production, and sSPC SUVs lost their ability to promote IL-4 expression in the presence of LPS, probably because IL-4 is an anti-inflammatory cytokine and its expression is inhibited in the presence of a pro-inflammatory stimulus such as LPS.

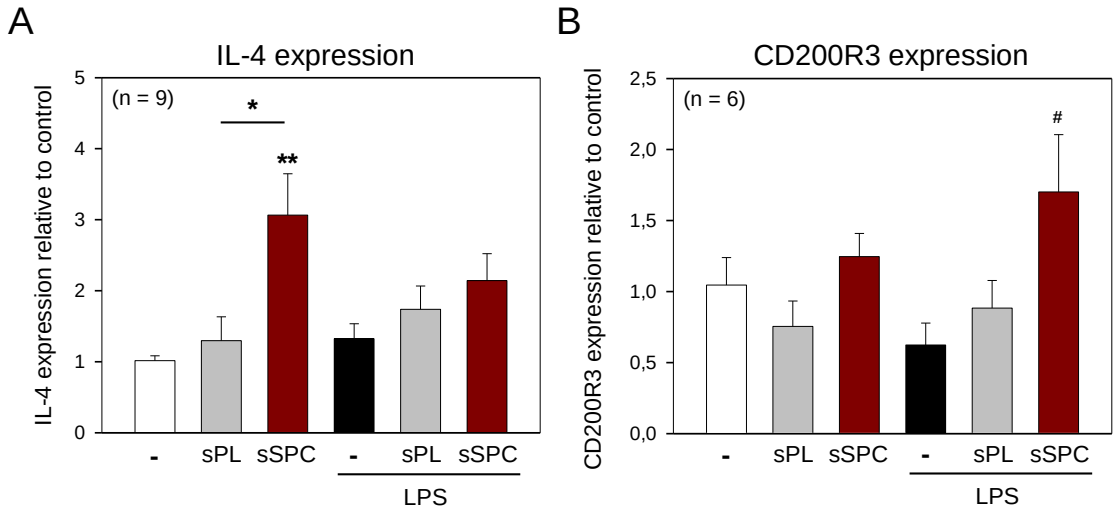


Figure 14: sSPC increases IL-4 (A) and CD200R3 (B) expressions. MH-S cells were preincubated with 250 µg/ml sPL or sSPC SUVs for 18 hours and stimulated with LPS (1 µg/ml) for 1 hour. RNA was then extracted for qPCR. Data are expressed as mean fold increases in RNA arbitrary units related to control cells ± SE (*$p<0.05$, **$p<0.01$ vs control or between indicated groups; # $p<0.05$ vs LPS).

CD200R3 is a member of the CD200 receptor family which comprises CD200R1, R2, R3, R4 and R5 in mice (Wright et al. 2003; Gorczynski et al. 2004b). The best characterized member of this family is CD200R1, which is expressed on resting AMs and can be induced by different pulmonary pathogens. Its ligand is CD200, which is found in many cell types, including airway epithelial cells (Snelgrove et al. 2008; Goulding et al. 2011). The interaction between CD200R1 and CD200 elicits immunoregulatory events in the cell expressing CD200R1 (Zhang et al. 2004). CD200R3 has also been proved to bind to CD200 (Gorczynski et al. 2004a), however, the signals transduced are less clear. It seems that CD200R3 activation causes degranulation in mast cells and IL-4 production in basophils (Kojima et al 2007). Thus, CD200R3 is involved in the development of

alternative immune responses. As we can see in figure 14B, CD200R3 expression is not influenced by the preincubation with surfactant vesicles in the absence of LPS and do not vary after the stimulation with LPS alone. Nevertheless, preincubation of the cells with sSPC SUVs followed by stimulation with LPS induces an increase in CD200R3 expression.

These results proved that the presence of SP-C in internalized synthetic surfactant vesicles specifically up-regulated the expression of IL-4 and CD200R3. Considering that both proteins are involved in the development of alternative immune responses, we tried to study the effect of internalized sSPC vesicles on the expression of other M2 markers or proteins induced by IL-4 or IL-13 like arginase I, chitinase 3-like-3 (Ym1), carbonic anhydrases 3 and 5a, and autotaxin (Scotton et al. 2005; Nair et al. 2006; Murray and Wynn 2011). However, we could not detect mRNA production for these proteins in MH-S AMs (data not shown).

On the other hand, iNOS is considered to be a marker of M1 or classically activated macrophages (Murray and Wynn 2011). Its expression can be induced by pro-inflammatory cytokines and other agents including LPS. Once expressed, the high amounts of NO produced by this enzyme can have beneficial microbicidal, antiviral, antiparasital and antitumoral effects (Pautz et al. 2010). The results concerning iNOS production in MH-S AMs are represented in figure 15. Basal iNOS mRNA and protein levels were almost undetectable and were not influenced by synthetic surfactant vesicles. As expected, stimulation of the cells with LPS induced iNOS production both at mRNA and protein levels. Preincubation of the cells with sSPC SUVs for 18 hours significantly increased LPS-elicited iNOS production, whereas sPL SUVs failed to show any modulatory effect.

Altogether, these findings demonstrated that, in addition to synthetic surfactant lipid component, internalized recombinant human SP-C present in sSPC membranes exerts an immunomodulatory effect on MH-S AMs, since it up-regulated basal IL-4 expression and LPS-elicited CD200R3 expression and iNOS production.

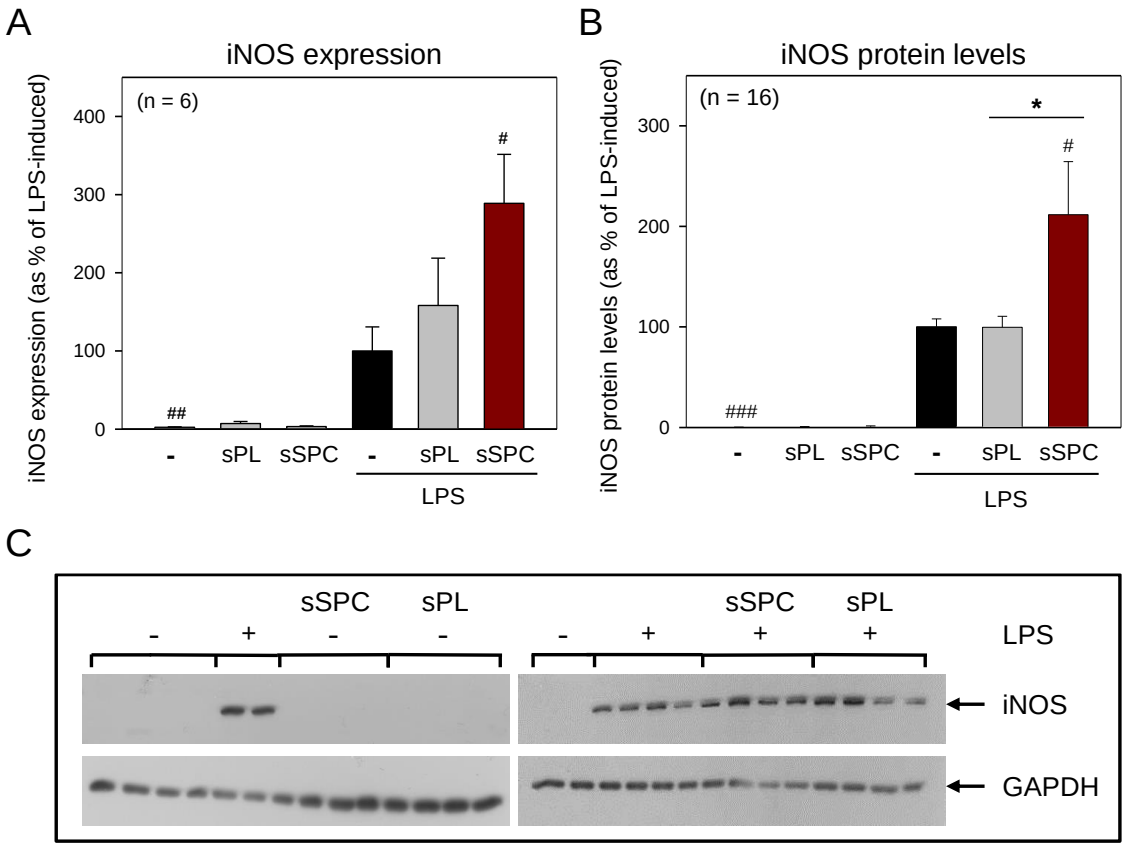


Figure 15: sSPC increases LPS-induced iNOS production. MH-S cells were preincubated with 250 μg/ml sPL or sSPC SUVs for 18 hours and stimulated with LPS (1 μg/ml) for 1 (A) or 6 (B) hours. (A) iNOS mRNA production was measured by qPCR. Data are expressed as percentages of LPS-induced iNOS expression ± SE. (B) iNOS protein levels were detected by western-blot and quantified by densitometry. LPS-induced iNOS protein level in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (C) Representative iNOS western-blot in MH-S cells treated as described in B. (*p<0.05 between indicated groups; #p<0.05, ##p<0.01, ###p<0.001 vs LPS).

5. DISCUSSION

In this work, we have used a synthetic surfactant composed of DPPC, POPG, PA and recombinant human SP-C to examine whether internalized surfactant vesicles were able to modify the activation state of MH-S murine AMs after being stimulated with bacterial LPS. The immunoregulatory properties exerted by this synthetic surfactant after being

endocytosed by AMs have never been studied before. Our results provide evidence that both human recombinant SP-C and the lipid component present in synthetic surfactant membranes exert different intracellular immunomodulatory actions once internalized by MH-S cells, balancing the expression of pro- and anti-inflammatory mediators and limiting LPS-induced pro-inflammatory response. Furthermore, sPL-induced immunoregulation was proved to be mediated at least in part by GSK3 activation.

In the first chapter of this thesis, we demonstrated that synthetic surfactant SUVs were endocytosed by MH-S cells by a clathrin-dependent mechanism. This process began almost ten minutes after the addition of the vesicles, and increased as the incubation time augmented. In this second chapter, we proved that surfactant MLVs were not internalized by MH-S AMs to the same extent than SUVs (figures 2 and 3). This finding was consistent with previous knowledge about surfactant metabolism in the alveolar space. Surfactant is secreted as large aggregates that have high surface activity and adsorb very rapidly to the air-liquid interface. Nevertheless, surface compression and expansion cycles generate small vesicles that have poor surface activity and are endocytosed by AMs and type II pneumocytes in order to assure its degradation and recycling (Casals and Cañadas 2012; Agassandian and Mallampalli 2013). Synthetic surfactant SUVs are more similar to natural surfactant small aggregates and therefore can be internalized by MH-S cells to a higher extent than MLVs. For this reason, only synthetic surfactant SUVs were used in the rest of experiments, in order to guarantee their endocytosis by MH-S murine AMs.

Internalized synthetic surfactant lipids were demonstrated to exert an immunomodulatory action on MH-S cells in the absence or presence of LPS. As we have already explained, the interaction of LPS with its receptor complex in the surface of macrophages triggers the activation of different signaling cascades. Among those are the pathways involving PI3K and Akt (O'Toole and Peppelenbosch 2007); NF-κB transcription factor; and ERK, JNK and p38 MAPKs (reviewed in Bode et al. 2012). sPL SUVs inhibited LPS-induced NF-κB activation (figure 8A), as well as basal and LPS-elicited Akt phosphorylation and activation (figure 8B). However, they showed no modulatory effect on LPS-induced phosphorylation of ERK and p38 MAPKs (figure 7). The mechanism underlying these results remains unknown.

Previous reports have studied the effect of DPPC vesicles on LPS-elicited response after being taken up by different cells. DPPC was proved to inhibit LPS-induced IL-8 production in A549 lung epithelial cells by blocking TLR4 translocation into lipid raft domains (Abate et al. 2010). The fact that internalized synthetic surfactant lipids down-regulated the activation of PI3K/Akt and NF-κB pathways but not the phosphorylation of ERK and p38 MAPKs allowed us to conclude that their immunoregulatory action must take place downstream of the TLR4/MD2 receptor complex. Thus, a direct effect on TLR4 translocation can be ruled out. Nevertheless, our results agree with other studies made by the same group showing that DPPC attenuated LPS-induced respiratory burst in a human monocytic cell line without affecting ERK or p38 MAPK signaling (Tonks et al. 2001). Instead, they demonstrated that this effect was mediated by the down-regulation of PKCδ isoform (Tonks et al. 2005).

To our knowledge, the inhibitory action of internalized surfactant lipids on the activation of the PI3K/Akt pathway has never been reported before. Class I PI3Ks generate phosphatidylinositol-3,4,5-triphosphate and indirectly phosphatidylinositol-3,4-biphosphate, which bind to the pleckstrin homology domain of Akt, promoting its phosphorylation and activation by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) (Vanhaesebroeck et al. 2012). Our results do not allow us to distinguish if the attenuation of basal and LPS-elicited Akt activation is the result of a down-regulation of class I PI3K, PDK1 or mTORC2 activities. Further research must be conducted in order to elucidate the mechanism underlying this effect. However, we have also demonstrated for the first time that internalized sPL SUVs promoted the activation of GSK3 in the absence or presence of LPS (figure 9), probably as a consequence of the inhibition of Akt. Interestingly, this finding represents a link between our work and the results obtained by Tonks et al. showing that DPPC attenuated LPS-induced respiratory burst in a human monocytic cell line via downregulation of PKCδ isoform (Tonks et al. 2005), because GSK3 has been shown to inhibit PKCδ activity (Wang et al. 2011). Moreover, as GSK3 has been reported to regulate a variety of signaling proteins and transcription factors (Hazeki et al. 2007; Wang et al. 2011), we hypothesized that sPL-induced modulation of the expression of pro- and anti-inflammatory markers could be mediated by GSK3 activation.

In fact, internalized synthetic surfactant lipids were proved to regulate the expression or production of several pro- and anti-inflammatory mediators. First of all, they inhibited LPS-elicited TNF-α expression and release (figure 4). TNF-α is a cytokine commonly produced by LPS-stimulated macrophages (Bode et al. 2012). It is also considered to be a powerful pro-inflammatory agent and a master-regulator of macrophage function and pro-inflammatory cytokine production. It induces an autocrine loop essential for sustaining inflammation and priming macrophages for enhanced responses to subsequent challenges (Parameswaran and Patial 2010). Hence, such a big decrease in TNF-α production (78 ± 3%) represents a strong limitation in the pro-inflammatory response to LPS. Interestingly, we suggested that internalized sPL SUVs could be regulating additional posttranscriptional steps apart from TNF-α mRNA synthesis, since the decrease in TNF-α secretion (figure 4B) was much more pronounced than the attenuation of its expression (figure 4A). In fact, ERK and p38 activation has been reported to be essential for the transport of TNF-α transcript from the nucleus to the cytoplasm and for the stabilization of this transcript, respectively (Bode et al. 2012). Here, ERK and p38 activities were not affected by internalized sPL SUVs (figure 7). However, TNF-α secretion is regulated by different proteins including one isoform of class I PI3Ks (Bode et al. 2012), which could be inhibited by internalized surfactant lipids, as we have discussed before.

Additionally, internalized synthetic surfactant lipids were also proved to reduce basal and LPS-induced IL-10 release (figure 6). IL-10 is an anti-inflammatory and immunosuppressive cytokine, produced by macrophages under steady stage and induced after the stimulation with pro-inflammatory agents. It is a key mediator of the resolution phase of inflammation (Murray and Wynn 2011; Bode et al. 2012). Furthermore, endocytosed sPL SUVs increased LPS-elicited expression of IRF1, COX2, CXCL10, CXCL11, CD80 and SOCS 3 (figure 10). Even though IRF1, CXCL10, CXCL11, and CD80 are considered to be pro-inflammatory markers (Widney et al. 2000; Mantovani et al. 2004; Ozato et al. 2007; Liu et al. 2011), COX2 has also been proved to play an anti-inflammatory role (Tsatsanis et al. 2006) and the effect of SOCS 3 is controversial and cannot be catalogued as a pro- or anti-inflammatory marker (Bode et al. 2012).

In order to test whether GSK3 activation was involved in the immunoregulatory action of endocytosed sPL SUVs, GSK3 was inhibited with lithium chloride added to the cells for

one hour after the preincubation with sPL SUVs and before the stimulation with LPS. LPS-induced release of TNF-α and IL-10 and expression of IRF1, COX2, CXCL10, CXCL11 and CD80 were decreased in the presence of lithium (figures 11, 12 and 13). This can be due to the inhibition of GSK3, or to the inhibition of other lithium-sensitive enzymes. In fact, lithium has been reported to inhibit inositol monophosphatase, inositol polyphosphate 1-phosphatase, bisphosphate nucleotidase, and phosphoglucomutase enzymes, among others (Quiroz et al. 2004). Moreover, its toxicity has been proved to be related to its deleterious effect on RNA processing in yeast (Dichtl et al. 1997). Nevertheless, inhibition of GSK3 activity suppressed the reduction of IL-10 release and the increase of LPS-elicited expression of IRF1, COX2, CXCL10, CXCL11 and CD80 induced by sPL (figures 12 and 13). Furthermore, it also decreased the inhibitory effect of sPL SUVs on LPS-elicited TNF-α release (figure 11). This effect was not completely abrogated in the presence of lithium, probably because TNF-α secretion is regulated by other proteins different from GSK3, including one isoform of class I PI3Ks (Bode et al. 2012). These proteins could be inhibited by internalized sPL SUVs even in the presence of lithium. Altogether, these findings demonstrated for the first time that the immunoregulatory action of internalized synthetic surfactant lipids in AMs is mediated at least in part by GSK3 activation.

The effect of the PI3K/Akt/GSK3 pathway on the regulation of the expression of immune related genes is very complex and seems to be cell-specific (Hazeki et al. 2007). A report demonstrated that GSK3 was able to modulate the expression of pro- and anti-inflammatory cytokines in LPS-stimulated monocytes through a mechanism that involved a molecular competition between CREB and NF-κB transcription factors for the co-activator of transcription CBP (CREB-binding protein). Since GSK3 inactivates CREB, GSK3 inhibition augmented the nuclear levels of CREB but had no discernible effect on NF-κB p65 levels. Both proteins have the same binding site on CBP. Therefore, GSK3 inhibition promoted the association of CREB with CBP, increasing the transcription of CREB-dependent genes while attenuating NF-κB-dependent expression of pro-inflammatory markers (Martin et al. 2005).

This mechanism could explain the immunoregulation exerted by internalized synthetic surfactant lipids. sPL SUVs inhibit Akt activity (figure 8B) and promote GSK3 activation (figure 9), both in the absence or presence of LPS. GSK3 activation could subsequently

attenuate CREB-dependent transcription. Although a variety of transcription factors have been shown to regulate IL-10 and TNF-α expression, the activation of CREB seems to be essential for IL-10 and TNF-α production in LPS-stimulated macrophages (Bode et al. 2012). This would be consistent with the reduction of IL-10 and TNF-α production in MH-S cells preincubated with sPL SUVs (figures 6 and 4). Moreover, NF-κB activation has been proved to be involved in the transcription of COX2 (Tsatsanis et al. 2006), CXCL10 (Liu et al. 2011), CXCL11 (Rani et al. 2001; Yang et al. 2007) and CD80 (Lim et al. 2005). LPS-elicited IRF1 expression depends on IRF3 activation, which also requires NF-κB and CBP to activate the transcription of interferon-inducible genes (Honda et al. 2006; Lu et al. 2008). Even though sPL SUVs have been shown to inhibit LPS-elicited IκB-α phosphorylation and subsequent NF-κB activation (figure 8A), GSK3 activation could increase free CBP nuclear levels, promoting an increase in the transcription of NF-κB-dependent genes. This would explain sPL-induced up-regulation of the expression of IRF1, COX2, CXCL10, CXCL11 and CD80 in LPS-stimulated MH-S cells (figure 10). This possible mechanism of action of internalized synthetic surfactant lipids is summarized in figure 16, although further experiments must be conducted in order to confirm it.

Concerning human recombinant SP-C, its presence in internalized synthetic surfactant membranes increased specifically basal IL-4 expression (figure 14A) and LPS-elicited CD200R3 expression (figure 14B) and iNOS production (figure 15). The mechanism that mediates SP-C immunoregulatory action after being endocytosed by AMs is currently not known. Our findings suggest that SP-C could up-regulate the activation of one or more signaling pathways. As mentioned before, IL-4 and CD200R3 are involved in the development of alternative immune responses (Kojima et al 2007; Galli et al. 2011; Murray and Wynn 2011) whereas iNOS is considered to be a marker of M1 or classically activated macrophages (Murray and Wynn 2011). The cellular pathways and transcription factors that activate the expression of M1 and M2 markers are generally very different. The exact conditions causing CD200R3 induction have not been yet elucidated. However, the Wnt/β-catenin pathway has been reported to be involved in the regulation of the expression of both IL-4 and iNOS (Pautz et al. 2010; Maier et al. 2012). In the absence of a Wnt ligand, β-catenin undergoes proteosome-dependent degradation. Upon binding of a Wnt ligand to its receptor, this degradation is inhibited and free β-catenin accumulates and translocates into

the nucleus, where it associates with T-cell factor (TCF) proteins, activating the transcription of Wnt target genes (reviewed in Sokol 2011). It has recently been demonstrated that TCF1 and β-catenin stimulate IL-4 expression in human $CD4^+$ T cells (Yu et al. 2009). Additionally, iNOS expression was activated by the Wnt signaling pathway in human cancer cell lines and primary human and rodent hepatocytes, and the binding of β-catenin and TCF4 increased the activity of iNOS promoter (Du et al. 2006). Taking this into consideration, the possible effect of internalized SP-C on the activation of the Wnt/β-catenin pathway could be examined in future experiments.

The critical role played by SP-C in the regulation of the activation state of AMs has already been reported in studies made with SP-C-deficient mice. The lack of SP-C was associated with increased pulmonary inflammation and reduced ability of AMs to clear pathogens during bacterial and virus infections (Glasser et al. 2008; Glasser et al. 2009; Glasser et al. 2013a). In this context, SP-C-induced increase in the production of iNOS could be advantageous, since NO has been proved to have beneficial microbicidal and antiviral effects (Pautz et al. 2010).

Altogether, these results indicate that both human recombinant SP-C and the lipid component present in internalized synthetic surfactant membranes modify the activation state of LPS-stimulated MH-S murine AMs. The physiological relevance of these findings is linked to the fact that the immune response has been shown to be organ-specific (Raz 2007). The lungs are continuously exposed to a diverse array of inhaled inorganic particles, allergens and pathogens, and an excessive inflammatory response could damage alveolar tissues. In this context, lung microenvironment has been shown to induce a unique phenotype in AMs, different from that of blood monocytes that enter the lungs during inflammatory processes and have been reported to present more pro-inflammatory features (Martin and Frevert 2005; Holt et al. 2008). Understanding the mechanism that regulates the activation state of AMs may serve to improve the therapies used to treat lung diseases, especially those linked to dysregulated immune responses.

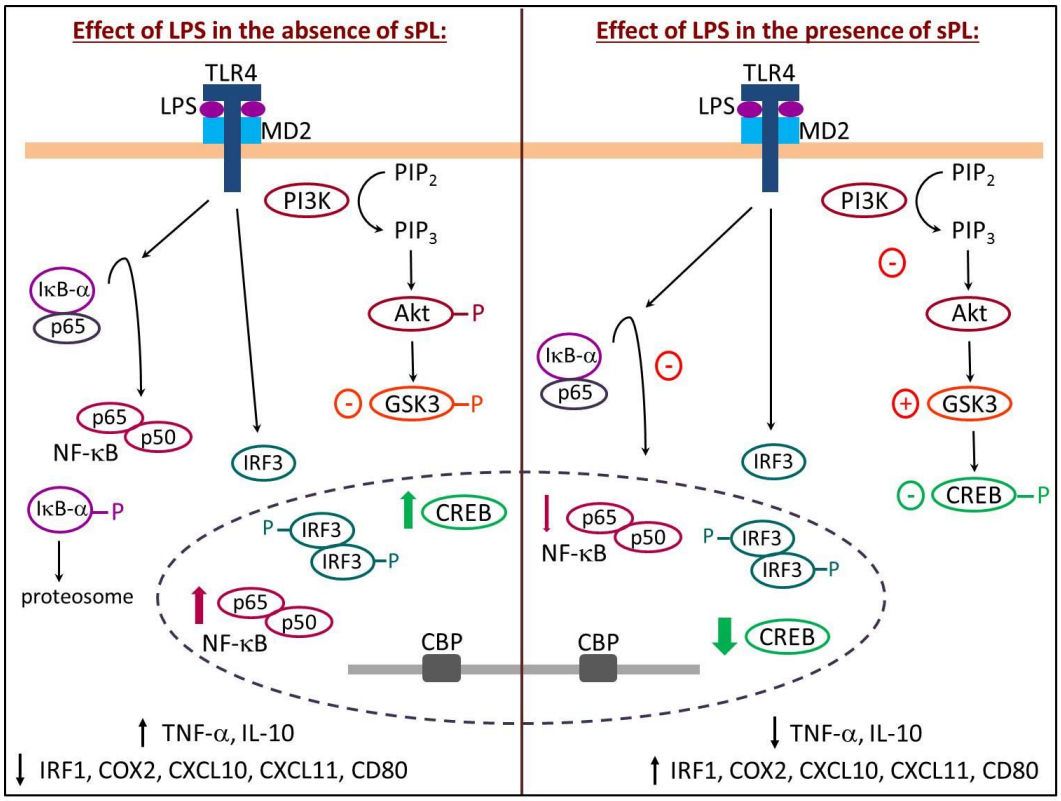


Figure 16: Possible mechanism for the immunoregulatory action of internalized synthetic surfactant lipids on the response of AMs to LPS. (Some signaling pathways activated by LPS, such as the MAPK pathway, have not been depicted here to simplify the figure).

Left panel**:** When LPS molecules interact with the TLR4/MD2 receptor complex in the surface of AMs, different signaling pathways are activated. Firstly, IκB-α is phosphorylated and subsequently proteosome-degraded, allowing NF-κB activation. Secondly, class I PI3Ks are activated and promote Akt phosphorylation and GSK3 inhibition. Additionally, IRF3 and CREB transcription factors are also activated and together with NF-κB, they migrate into the nucleus. There, NF-κB and CREB compete for the co-activator of transcription CBP in order to initiate the transcription of their target genes. Moreover, NF-κB and CBP are necessary for IRF3-dependent activation of interferon-inducible gene transcription.

Right panel: Internalized surfactant lipids inhibit IκB-α phosphorylation and NF-κB activation, as well as Akt phosphorylation. As a consequence, they promote the activation of GSK3, which in turn inhibits CREB. This results in lower IL-10 and TNF-α expression. As NF-κB activation is not completely suppressed, the reduction of CREB nuclear levels promotes the binding of NF-κB to CBP, increasing the expression of NF-κB- and IRF3-dependent genes, such as IRF1, COX2, CXCL10, CXCL11 and CD80.

CHAPTER 3

Immunomodulatory effect of internalized synthetic surfactant vesicles on A549 alveolar epithelial cells infected with respiratory syncytial virus

1. ABSTRACT

Respiratory syncytial virus (RSV) causes life-threatening bronchiolitis and pneumonia in premature infants, the elderly and immunosuppressed patients. Surfactant components have been shown to play a protective role in the regulation of the immune response against this virus. However, their antiviral action after being endocytosed by type II alveolar epithelial cells (AECs) has never been studied before. The purpose of this study was to investigate the intracellular immunoregulatory functions of internalized vesicles of a synthetic surfactant based on recombinant human surfactant protein C (SP-C) on the immune response of A549 human AECs infected with human RSV. SP-C protein levels were increased in A549 cells after their preincubation for 18 hours with synthetic surfactant, suggesting that surfactant vesicles had been taken up by the cells. Internalized synthetic surfactant vesicles did not inhibit virus replication. Nevertheless, they reduced RSV-induced expression of inflammatory markers as well as RSV-elicited activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) and the phosphatidylinositol-3-kinase (PI3K) downstream effector kinase Akt. However, they did not modify the activation of the MAPK p38 or the nuclear factor-κB (NF-κB) canonical pathway. Surfactant lipid component was demonstrated to be responsible for the down-regulation of RSV-induced ERK activation and expression of interferon-stimulated gene 15 (ISG15) and retinoic acid-inducible gene I (RIG-I). The possible immunomodulatory role of internalized SP-C in RSV-infected A549 cells remains to be determined. Altogether, these results indicate that internalized synthetic surfactant vesicles exert an immunoregulatory action on A549 AECs infected by RSV. Understanding this anti-inflammatory mechanism may be important in order to design new antiviral therapies based on pulmonary surfactant components.

2. INTRODUCTION

RSV is an enveloped non-segmented negative-sense RNA virus that belongs to the *Paramyxoviridae* family, subfamily *Pneumovirinae*. It is a common and highly contagious respiratory pathogen, considered to be the most frequent cause of lower respiratory tract illness among infants worldwide. Virtually, all children by the age of 2 have been infected at least once by RSV. Symptoms from infection vary from mild manifestations in healthy

adults to life-threatening bronchiolitis and pneumonia in high risk individuals, including premature infants, the elderly and immunosuppressed patients. Moreover, severe RSV disease early in life is frequently followed by lingering abnormalities in airway function, such as recurrent wheezing, allergic sensitization and development of asthma. Reinfections can occur multiple times throughout life, suggesting that the adaptive immunity to RSV is incomplete. Furthermore, no reliable vaccine or antiviral therapies against this virus are currently available. Thus, the development of new pharmacological tools to control RSV infection remains an urgent challenge (reviewed in Collins and Melero 2011).

Airway epithelial cells are the main target of RSV in the respiratory tract, but this virus can also infect other structural and resident immune cells (Lotz and Peebles 2012). Viral components are detected by pattern recognition receptors (PRRs) expressed by these cells, including RIG-I, Toll-like receptor (TLR)3 (Liu et al. 2007), TLR4 (Kurt-Jones et al. 2000), TLR2, TLR6 (Murawski et al. 2009) and TLR7 (Lukacs et al. 2010), triggering the initiation of the innate immune response against the virus (Bueno et al. 2011). RSV induces a strong pro-inflammatory response, which promotes virus clearance but can also be detrimental to the host. In fact, inefficient immunoregulation has been proved to increase disease severity following RSV infection (Lotz and Peebles 2012).

In the alveolar space, RSV encounters pulmonary surfactant components, which represent the first mean of interaction between the virus and the innate pulmonary defense (Barreira et al. 2011). Pulmonary surfactant is a complex network of extracellular membranes synthesized and secreted into the alveolar space by type II AECs. It consists of approximately 90% of lipids (mainly phospholipids) and contains four associated proteins (surfactant proteins A, B, C and D (SP-A, SP-B, SP-C and SP-D)) (Agassandian and Mallampalli 2013). Its main function is to reduce the surface tension at the air-liquid interface in order to prevent the lungs from collapsing at the end of expiration. Surfactant deficiency in immature lungs is the main cause of neonatal respiratory distress syndrome (RDS) (Whitsett and Weaver 2002). In addition to its biophysical relevance, surfactant has also been recognized to play an essential role in the immune host defense of the lungs (Chroneos et al. 2010; Glasser and Mallampalli 2012).

In fact, the protective role of surfactant collectins SP-A and SP-D against RSV has been proved in studies performed with SP-A and SP-D knock-out mice. Both proteins were

shown to bind to RSV F and G glycoproteins, to reduce infectivity and inflammation *in vivo* and to increase viral clearance *in vitro* and *in vivo* (Ghildyal et al. 1999; Hickling et al. 1999; LeVine et al. 1999; Barr et al. 2000; Hickling et al. 2000; LeVine et al. 2004). Additionally, palmitoyloleoylphosphatidylglycerol (POPG), a surfactant anionic phospholipid, was demonstrated to suppress RSV-induced inflammation and infection *in vitro* and *in vivo* by directly binding to RSV and blocking viral attachment to epithelial cell surface, and to provide short-term prophylaxis against RSV infection in mice (Numata et al. 2010; Numata et al. 2013a; Numata et al. 2013b). Concerning SP-C, patients with mutations in the gene encoding this protein suffer recurrent neonatal and childhood pulmonary infections by influenza virus and RSV (Chibbar et al. 2004; Bullard and Nogee 2007). SP-C knock-out mice were proved to be susceptible to RSV infection, with decreased viral clearance and more robust and sustained pulmonary inflammation (Glasser et al. 2009). Furthermore, genetic replacement of SP-C in these mice reduced RSV-elicited inflammation and restored viral clearance (Glasser et al. 2013a). However, the mechanism underlying the antiviral action of SP-C is not fully elucidated. Understanding how surfactant components influence the immune response against RSV is essential to develop new therapeutic strategies.

In the alveolar space, surfactant is endocytosed by alveolar macrophages (AMs) and type II pneumocytes in order to assure its degradation and recycling (Agassandian and Mallampalli 2013). In the second chapter of this thesis, internalized vesicles of a synthetic surfactant based on recombinant human SP-C were shown to modify the activation state of AMs and to limit lipopolysaccharide (LPS)-induced pro-inflammatory response. This synthetic surfactant is composed of dipalmitoylphosphatidylcholine (DPPC), POPG, palmitic acid (PA) and recombinant human SP-C. The effect of these surfactant components once endocytosed by type II AECs on their immune response against RSV has not been studied yet.

Taking this into consideration, the purpose of this study was to investigate the intracellular immunoregulatory functions of internalized synthetic surfactant vesicles on the immune response of human AECs (A549 cells) infected with human RSV. More specifically, the aims were to find out which of its components exerted these effects and which signaling pathways were involved in it.

3. EXPERIMENTAL DESIGN

As we have already mentioned in this thesis, the synthetic surfactant based on recombinant human SP-C contains 98% of lipids by weight (DPPC/POPG/PA 2.3:1:0.16 w/w) and 2% of human recombinant SP-C. SP-C is a small hydrophobic peptide with a α-helix segment which adopts a transmembrane orientation, and a more polar N-terminal segment with two palmitoylated cysteines (Vandenbussche et al. 1992a). However, recombinant SP-C is not palmitoylated (see chapter 1 section 3). Surfactant small unilamellar vesicles (SUVs) were prepared as described in materials and methods.

To examine the effect of internalized synthetic surfactant vesicles on the immune response of A549 AECs to RSV, these cells were preincubated with synthetic surfactant membranes containing human recombinant SP-C (sSPC), or surfactant membranes without SP-C (sPL), for 18 hours (figure 1). After that, they were mock-infected (with sucrose) or infected with human RSV (Long strain) at a multiplicity of infection (moi) of 3 plaque-forming units (pfu) per cell.

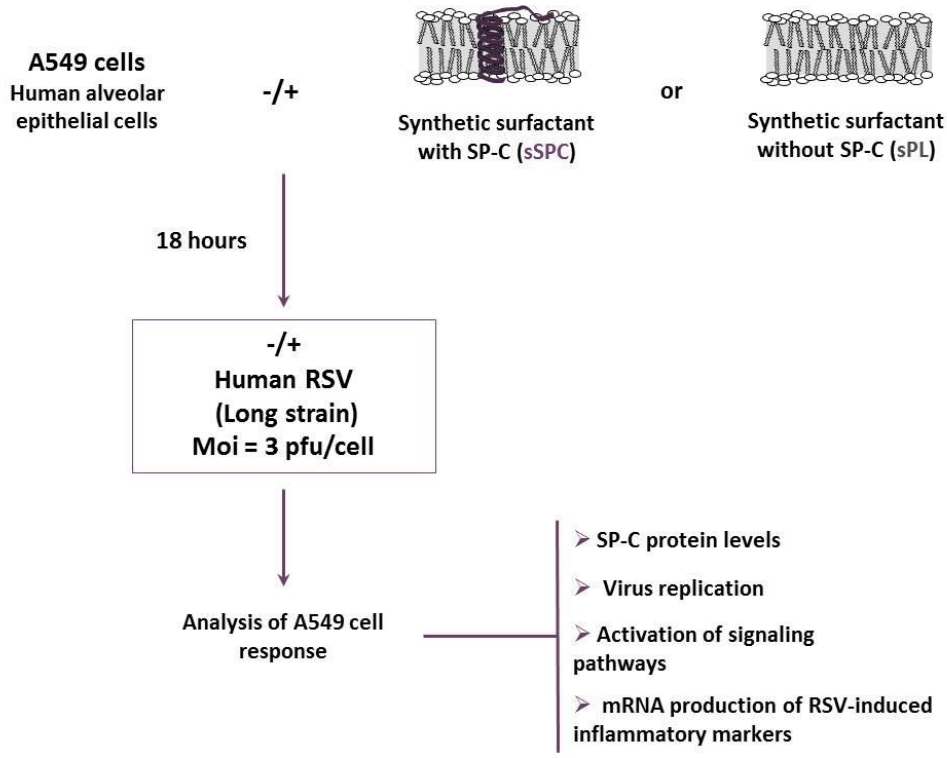


Figure 1: Experimental design used in chapter 3.

SP-C protein levels in A549 cells were detected by western-blot to confirm the internalization of synthetic surfactant vesicles. Virus replication was evaluated determining virus titers by plaque assay in layered HEp-2 cells, and quantifying mRNA levels of RSV N nucleoprotein by quantitative real-time polymerase chain reaction (qPCR). Expression of RSV-induced inflammatory markers was also determined by qPCR. Finally, activation of signaling pathways was analyzed by western-blot.

4. RESULTS

4.1 Recombinant human SP-C uptake by A549 AECs

In the alveolar space, surfactant is endocytosed by AMs and type II AECs by a mechanism that involves clathrin-mediated endocytosis accompanied by actin polymerization, in order to assure its degradation and recycling (Agassandian and Mallampalli 2013). Accordingly to this, synthetic surfactant SUVs were proved to be internalized by a clathrin-mediated mechanism by MH-S murine AMs (chapter 1). This process began almost ten minutes after the addition of the vesicles, and increased as the incubation time augmented.

A549 cells are human epithelial cells that retain morphological features of type II AECs (Lieber et al. 1976). For this reason, our first experiments were conducted to analyze if these cells were also able to internalize synthetic surfactant SUVs. A549 cells were preincubated for 18 hours with 100 or 250 μg/ml sSPC SUVs. This preincubation time was long enough to allow surfactant vesicle endocytosis by AMs (chapters 1 and 2). Cells were subsequently mock-infected (with sucrose) or infected with RSV for 24 hours. After that, SP-C protein levels were determined by western-blot. As we can see in figure 2, A549 cells did not express SP-C by themselves. Nevertheless, after the preincubation with sSPC SUVs for 18 hours, SP-C levels were increased in the cells both in the presence or absence of RSV. In mock-infected cells, SP-C protein levels augmented in a sSPC concentration-dependent way. However, SP-C protein levels were similar in RSV-infected cells that had been preincubated with sSPC SUVs at 100 and 250 μg/ml. In spite of this difference, these results indicated that recombinant human SP-C had been taken up by A549 AECs after their preincubation with synthetic surfactant SUVs for 18 hours, suggesting that sSPC

SUVs had also been internalized by the cells. Thus, we concluded that 18 hours was a time long enough to allow the internalization of synthetic surfactant SUVs by A549 AECs.

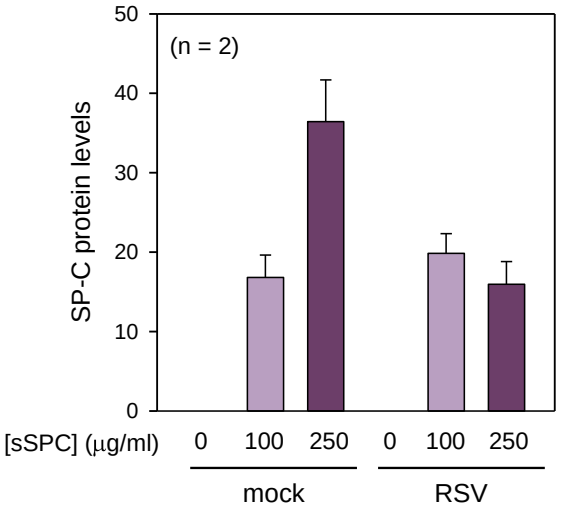


Figure 2: Human recombinant SP-C is taken up by A549 AECs after their preincubation with synthetic surfactant vesicles for 18 hours. A549 cells were preincubated with sSPC SUVs (100 or 250 μg/ml) for 18 hours and subsequently mock-infected (with sucrose) or infected with RSV (moi of 3 pfu/cell) for 24 hours. SP-C protein levels were detected by western-blot, and quantified by densitometry using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. Data are expressed as mean expression in arbitrary units ± standard error (SE).

4.2 Viral replication analysis

Our next assays were performed in order to study if internalized synthetic surfactant vesicles were able to inhibit viral replication in RSV-infected A549 AECs. These cells were preincubated for 18 hours with 250 μg/ml sSPC SUVs, and subsequently infected with RSV for different times. Virus titers in culture supernatants were determined by plaque assay in layered HEp-2 cells. Results are shown in table 1. Synthetic surfactant vesicles failed to reduce virus titers in culture supernatants of RSV-infected A549 cells.

Another method to evaluate RSV replication consists in quantifying mRNA levels of RSV N nucleoprotein, a protein which binds to viral RNA to generate the viral ribonucleoprotein complex (Tawar et al. 2009). RSV N nucleoprotein expression was

quantified by qPCR in A549 AECs previously preincubated for 18 hours with sSPC SUVs and subsequently infected with RSV for 24 hours. As we can see in figure 3, preincubation of the cells with synthetic surfactant vesicles did not decrease N mRNA production.

Altogether, these results indicated that internalized synthetic surfactant vesicles failed to show any modulatory effect on viral replication in RSV-infected A549 AECs.

Table 1: Virus titers in supernatants of A549 cells preincubated for 18 hours with 250 μg/ml sSPC SUVs and infected with human RSV (moi of 3 pfu/cell) for different times.

	t = 0	**t = 16h**	**t = 24h**	**t = 48h**
RSV	$5.25x10^3$	$6.75x10^3$	$1.1x10^4$	$1.64x10^6$
RSV + sSPC	$4.25x10^3$	$5.75x10^3$	$9.37x10^3$	$1.2x10^6$

Data from a representative experiment are expressed as virus titers in pfu/ml.

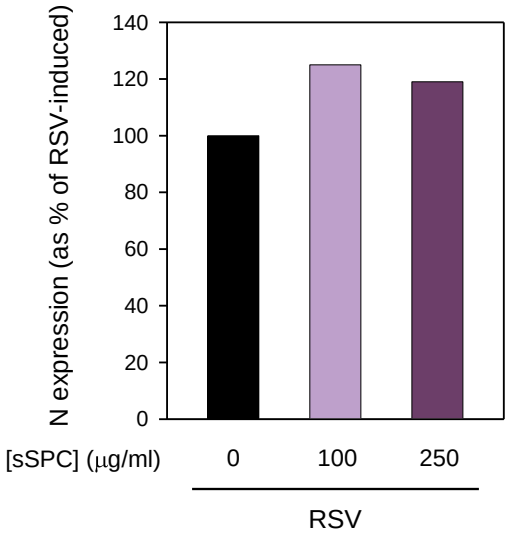


Figure 3: Internalized synthetic surfactant vesicles do not reduce the expression of RSV N nucleoprotein. A549 cells were preincubated with sSPC SUVs (100 or 250 μg/ml) for 18 hours, and subsequently infected with RSV (moi of 3 pfu/cell) for 24 hours. RNA was then extracted for qPCR. Data are expressed as percentages of RSV-induced expression in the absence of surfactant.

4.3 Inhibition of RSV-induced expression of inflammatory mediators

Internalized synthetic surfactant vesicles were proved to modulate the expression of different pro- and anti-inflammatory markers in LPS-stimulated MH-S murine AMs (chapter 2). For this reason, our next step consisted in analyzing if internalized synthetic surfactant vesicles were also able to regulate RSV-elicited expression of different inflammatory mediators in A549 AECs. These cells were preincubated with 250 μg/ml sSPC SUVs for 18 hours and subsequently mock-infected or infected with RSV for 24 hours. After that, the expressions of ISG15, interferon-induced protein with tetratricopeptide repeats (IFIT) 1, tumor necrosis factor α-induced protein 3 (TNFAIP3), RIG-I and TLR3 were quantified by qPCR.

All these inflammatory mediators play an important role in the immune response against RSV and other viral pathogens. ISG15 is an ubiquitin-like protein that is conjugated to numerous cellular and viral proteins and that exhibits a strong antiviral activity (Jeon et al. 2010; Skaug and Chen 2010). IFIT 1 is also an antiviral protein that reduces viral replication and binds directly to viral RNA (Zhou et al. 2013). TNFAIP3 is a cytoplasmic zinc finger protein with ubiquitin-modifying activity that inhibits the activation of NF-κB and interferon regulatory factor 3 (IRF3) transcription factors and cell death (Ma and Malynn 2012; Maelfait et al. 2012; Lai et al. 2013). RIG-I and TLR3 are PRRs that recognize double-stranded RNA (dsRNA). RIG-I is a cytosolic RNA helicase, whereas TLR3 is a type I transmembrane receptor localized in the cell surface or in endosome compartments (Yu and Levine 2011). Both have been demonstrated to be activated during RSV-infection in A549 AECs (Liu et al. 2007). The expression of these 5 molecules has been reported to be induced in RSV-infected A549 cells or MLE-15 cells (mouse type II AECs) (Liu et al. 2007; Martínez et al. 2007; Moore et al. 2008).

Results are depicted in figure 4. As expected, ISG15, IFIT 1, TNFAIP3, RIG-I and TLR3 expressions were induced in RSV-infected A549 AECs. Internalized sSPC SUVs did not modify the expression of these proteins in mock-infected cells. However, they significantly decreased RSV-elicited expression of the 5 proteins. This result demonstrated that internalized synthetic surfactant vesicles exerted an immunomodulatory effect on RSV-infected A549 AECs, inhibiting RSV-induced expression of inflammatory mediators.

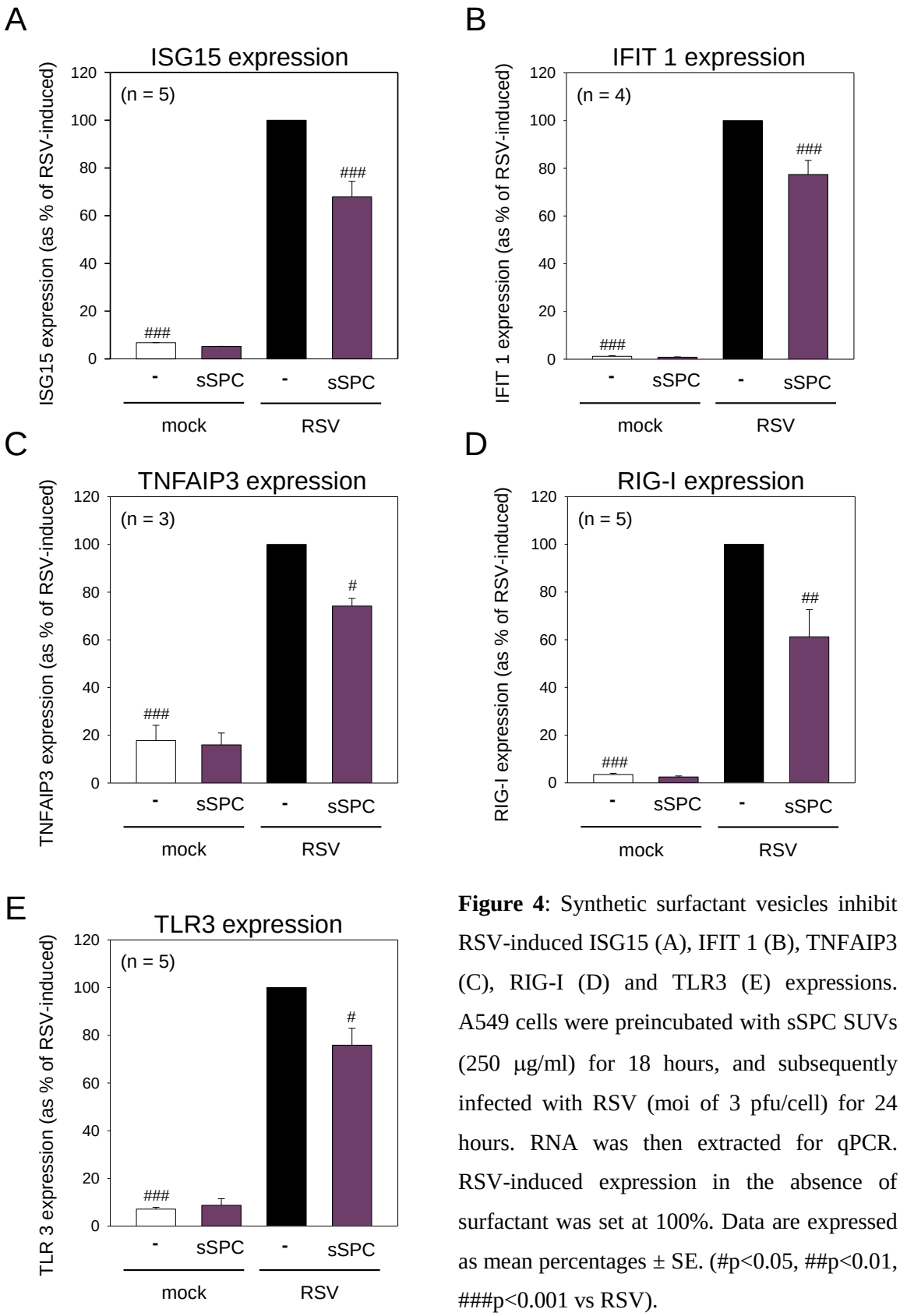


Figure 4: Synthetic surfactant vesicles inhibit RSV-induced ISG15 (A), IFIT 1 (B), TNFAIP3 (C), RIG-I (D) and TLR3 (E) expressions. A549 cells were preincubated with sSPC SUVs (250 μg/ml) for 18 hours, and subsequently infected with RSV (moi of 3 pfu/cell) for 24 hours. RNA was then extracted for qPCR. RSV-induced expression in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (#p<0.05, ##p<0.01, ###p<0.001 vs RSV).

In order to examine which of synthetic surfactant components was responsible for this immunoregulatory action, A549 cells were preincubated with sSPC or sPL SUVs for 18 hours and subsequently mock-infected or infected with RSV for 24 hours. Then, the expressions of ISG15 and RIG-I were quantified by qPCR (figure 5). Accordingly to the results shown in figure 4, ISG15 and RIG-I expressions were increased after the infection of the cells with RSV. Synthetic surfactant vesicles did not modify the expression of these molecules in mock-infected cells. Nonetheless, sPL and sSPC SUVs significantly down-regulated RSV-induced expression of ISG15 and RIG-I. Since this inhibitory effect was similar in the absence or presence of recombinant human SP-C in surfactant membranes, we concluded that the lipid component of internalized synthetic surfactant vesicles was responsible for the modulation of RSV-elicited expression of these 2 inflammatory markers.

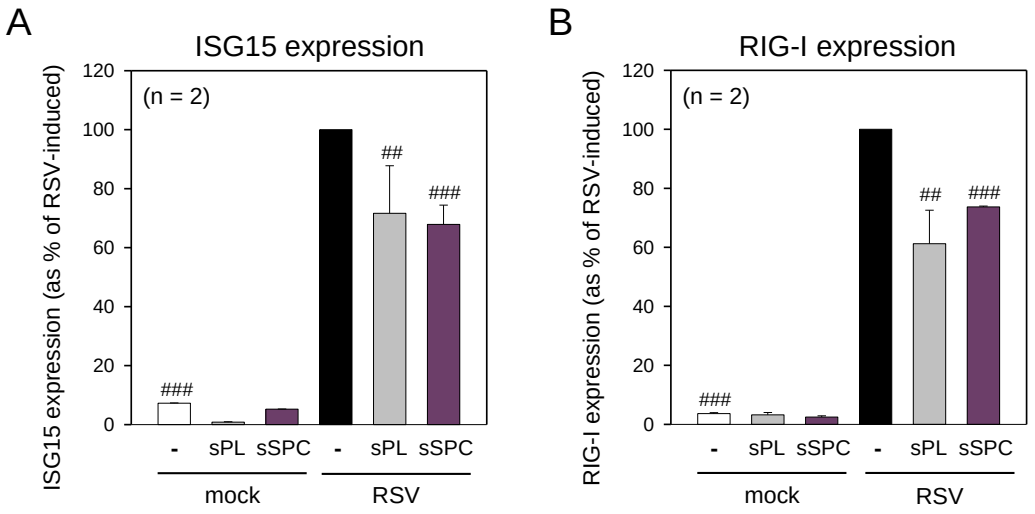


Figure 5: The inhibition of RSV-induced ISG15 (A) and RIG-I (B) expressions is exerted by synthetic surfactant lipids. A549 cells were preincubated with sSPC or sPL SUVs (250 μg/ml) for 18 hours, and subsequently infected with RSV (moi of 3 pfu/cell) for 24 hours. RNA was then extracted for qPCR. RSV-induced expression in the absence of surfactant was set at 100%. Data are expressed as mean percentages ± SE. (##p<0.01, ###p<0.001 vs RSV).

4.4 Inhibition of RSV-activated signaling pathways

After RSV components are recognized by cellular PRRs, different signaling cascades are activated. The activation of TLR3 and RIG-I by viral dsRNA or the activation of TLR4 by RSV F glycoprotein triggers the activation of ERK, p38 and c-Jun N-terminal kinase (JNK) MAPKs and the phosphorylation and nuclear translocation of IRF3 and IRF7 transcription factors, inducing the expression of type I interferons (IFN) and IFN-stimulated genes (Yu and Levine 2011; Newton and Dixit 2012). It also promotes the activation of NF-κB canonical pathway. In this pathway, NF-κB inhibitor protein IκB-α is phosphorylated and targeted for proteosomal degradation. As a result, Rel A (p65)/p50 complexes are released to enter the nucleus and activate NF-κB-dependent gene transcription (Yu and Levine 2011; Sun 2011; Newton and Dixit 2012). Additionally, RSV infection has also been proved to activate the PI3K/Akt pathway in A549 AECs (Thomas et al. 2002; Bitko et al. 2007).

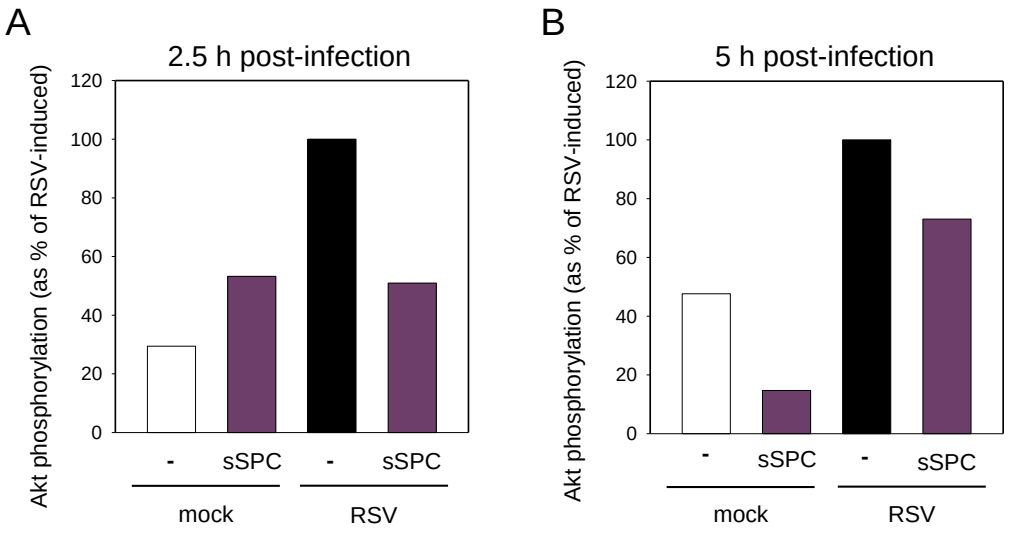


Figure 6: Synthetic surfactant vesicles inhibit RSV-induced Akt phosphorylation. A549 cells were preincubated with sSPC SUVs (250 μg/ml) for 18 hours, and subsequently infected with RSV (moi of 3 pfu/cell) for 2.5 (A) or 5 (B) hours. Akt phosphorylation was detected by western-blot with a phospho-specific antibody, and quantified by densitometry using total protein as loading control. Data are expressed as percentages of RSV-induced phosphorylation level in the absence of surfactant.

In order to analyze if internalized synthetic surfactant vesicles were able to inhibit RSV-induced activation of the PI3K/Akt pathway, A549 cells were preincubated with sSPC SUVs for 18 hours and subsequently mock-infected or infected with RSV for 2.5 or 5 hours. Then, the phosphorylation of the PI3K downstream effector kinase Akt was detected by western-blot. As we can see in figure 6, Akt phosphorylation was increased in A549 cells after 2.5 or 5 hours of infection with RSV. In mock-infected cells, sSPC SUVs increased or decreased Akt activation at 2.5 or 5 hours after sucrose addition, respectively. However, in RSV-infected cells, sSPC SUVs reduced Akt phosphorylation at 2.5 and 5 hours post-infection. Although their effect on mock-infected cells was controversial, internalized synthetic surfactant vesicles exerted an inhibitory action on the activation of the PI3K/Akt pathway in RSV-infected A549 AECs.

Additionally, the phosphorylation of ERK and p38 MAPKs and IκB-α was also analyzed in A549 AECs preincubated with synthetic surfactant vesicles for 18 hours and subsequently mock-infected or infected with RSV for 2.5 hours. In this case, sSPC and sPL SUVs were used, in order to determine the roles of SP-C and the lipid component present in surfactant membranes. As it is shown in figure 7B, RSV infection failed to induce the phosphorylation of p38 and IκB-α, and surfactant SUVs did not show any regulatory effect on their phosphorylation. Nevertheless, RSV infection elicited an increase in ERK phosphorylation levels. Surfactant vesicles did not modify ERK activation in mock-infected cells. However, sPL and sSPC SUVs inhibited ERK phosphorylation in RSV-infected A549 cells by 68% and 64%, respectively (figure 7A). Since this modulatory effect was similar in the presence or absence of recombinant human SP-C, we concluded that internalized synthetic surfactant lipid component was responsible for the inhibition of RSV-elicited ERK activation in A549 AECs.

Altogether, these results indicate that internalized synthetic surfactant vesicles are able to down-regulate RSV-induced activation of the PI3K/Akt pathway and ERK MAPK. However, they cannot modify the activation of the NF-κB canonical pathway or p38 MAPK. Moreover, synthetic surfactant lipid component was shown to be responsible for the inhibitory effect on RSV-induced ERK activation.

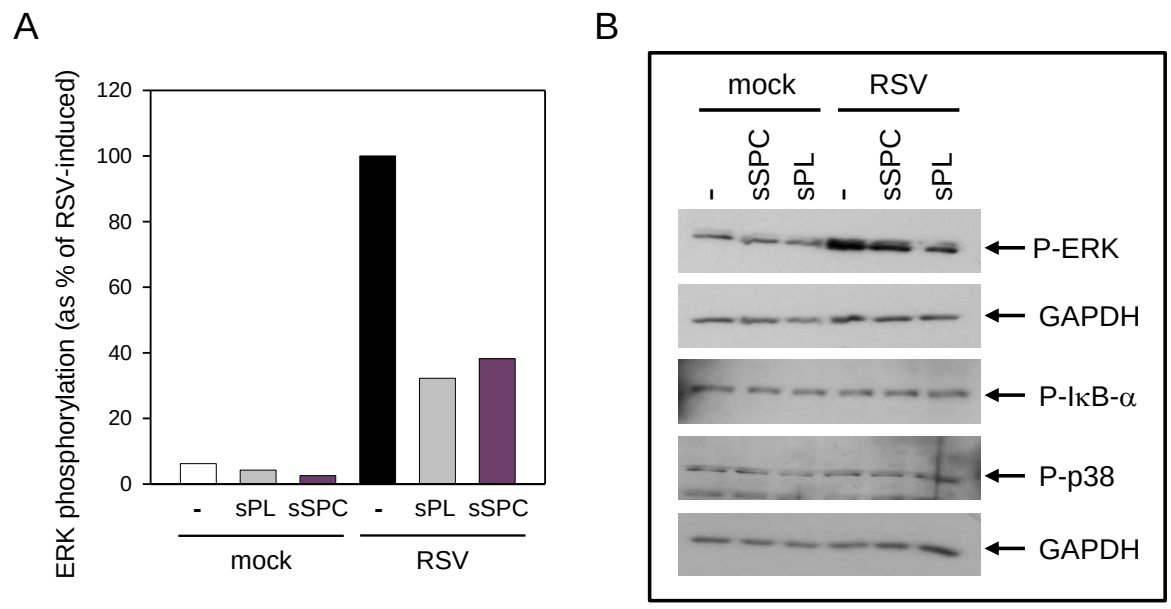


Figure 7: Surfactant lipids inhibit RSV-induced ERK but not p38 nor IκB-α phosphorylation. A549 cells were preincubated with sSPC or sPL SUVs (250 μg/ml) for 18 hours, and subsequently infected with RSV (moi of 3 pfu/cell) for 2.5 hours. ERK, p38 and IκB-α phosphorylations were detected by western-blot with phospho-specific antibodies, and quantified by densitometry using GAPDH as loading control. (A) ERK phosphorylation data are represented as percentages of RSV-induced phosphorylation level in the absence of surfactant. (B) Representative western-blot images of A549 cells treated as explained above.

5. DISCUSSION

The purpose of this study was to investigate the intracellular immunoregulatory functions of internalized vesicles of a synthetic surfactant based on recombinant human SP-C on the immune response of A549 AECs infected with RSV. Even though surfactant components have been proved to play a protective role against RSV, their effect on the immune response against this virus after being endocytosed by type II AECs has never been studied before. Our results provide evidence that internalized synthetic surfactant vesicles exert an immunomodulatory action on RSV-infected A549 AECs, since they inhibited RSV-induced expression of inflammatory mediators, as well as RSV-elicited activation of the PI3K/Akt pathway and ERK MAPK. Moreover, synthetic surfactant lipid component

was demonstrated to be responsible for the down-regulation of ISG15 and RIG-I expressions and ERK activation in RSV-infected cells.

A549 cells are human epithelial cells that retain morphological features of type II AECs (Lieber et al. 1976). They have been reported to synthesize and secrete saturated phosphatidylcholines, to contain multilamellar cytoplasmic inclusion bodies and to produce surfactant proteins, including SP-C (Lieber et al. 1976; Smith 1977; Vaporidi et al. 2005; Liu et al. 2012). However, A549 AECs used in this work did not express SP-C by themselves (figure 2). This highlights the high genetic and phenotypic variability that exists among cells of a same line. In the alveolar space, surfactant is endocytosed by AMs and type II AECs by a mechanism that involves clathrin-mediated endocytosis accompanied by actin polymerization, in order to assure its degradation and recycling (Agassandian and Mallampalli 2013). Accordingly to this, synthetic surfactant SUVs were proved to be internalized by a clathrin-mediated mechanism by MH-S murine AMs (chapter 1). Analysis of SP-C protein levels in A549 cells previously preincubated with sSPC SUVs for 18 hours and subsequently mock-infected or infected with RSV for 24 hours revealed that SP-C had been taken up by these cells (figure 2). Therefore, we concluded that 18 hours was a time long enough to allow the internalization of synthetic surfactant vesicles by A549 AECs. Confocal microscopy experiments should be conducted in order to determine the mechanism by which A549 cells accomplish this internalization.

Internalized synthetic surfactant vesicles were not able to inhibit viral replication in RSV-infected A549 AECs (table 1, figure 3). However, one component of synthetic surfactant, POPG, has been shown to block viral plaque formation *in vitro*, to suppress the expansion of plaques from RSV-pre-infected cells and to inhibit viral replication *in vivo*. Interestingly, in the experiments *in vitro*, POPG was added to the cells one hour before, simultaneously to, or 2 and 24 hours after RSV infection. In *in vivo* assays, the protective effect of POPG against RSV was maximal when the lipid was added simultaneously to or 45 minutes before RSV infection, while pretreatment times of 12, 8 or 4 hours were ineffective. This was due to the fact that POPG mechanism of action takes place in the extracellular medium, where POPG directly binds to RSV and blocks viral attachment to epithelial cell surface (Numata et al. 2010; Numata et al. 2013a; Numata et al. 2013b). Consequently, when POPG is internalized by alveolar cells, its inhibitory action on viral

replication disappears. This explains why POPG present in internalized synthetic surfactant vesicles is not able to reduce RSV replication in infected A549 AECs.

Airway epithelial cells are the first target of RSV in the respiratory tract and are responsible for the initiation of the immune response against the virus (Bueno et al. 2011). A correct immunoregulation is particularly important during the infection with RSV, because this virus has been proved to induce a strong pro-inflammatory response, which promotes virus clearance but can also be detrimental to the host. Moreover, inefficient immunoregulation has been proved to increase disease severity following RSV infection (Lotz and Peebles 2012). In fact, RSV disease arises from both direct viral cytopathic damage and the host immune response, being their relative contributions still controversial (Collins and Melero 2011).

In this regard, internalized synthetic surfactant vesicles have been demonstrated to exert an immunomodulatory action on RSV-infected A549 AECs, since they decreased RSV-elicited expression of ISG15, IFIT 1, TNFAIP3, TLR3 and RIG-I (figure 4). This inhibition was not complete, which could be beneficial since these molecules have been recognized to play an important role in the immune response against RSV and other viral pathogens. However, down-regulating their expression could also be advantageous. Firstly, ISG15 exerts its antiviral action by being conjugated to cellular and viral proteins, but it can also be released to the extracellular medium by lysis of infected cells. There, ISG15 has been shown to induce the synthesis and secretion of IFN-γ from B-cell-depleted lymphocytes, indicating that ISG15 can act as a pro-inflammatory cytokine (Recht et al. 1991; Jeon et al. 2010). Secondly, IFIT 1 has also antiviral activity, but its over-expression can inhibit virus-induced NF-κB and IRF3 activation and IFN-β production, impairing cellular antiviral response (Li et al. 2009; Zhou et al. 2013). Similarly, TNFAIP3-deficient mice have been proved to be protected against lethal influenza A virus infection, suggesting that inhibition of TNFAIP3 expression can be of interest in certain conditions (Maelfait et al. 2012). Finally, TLR3 and RIG-I are the main PRRs implicated in RSV recognition. Their activation triggers the expression of numerous pro-inflammatory chemokines and cytokines by AECs (Liu et al. 2007; Welliver 2008; Bueno et al. 2011). The modulation of their expression could therefore have a protective effect against RSV-induced inflammation.

RIG-I has been demonstrated to mediate RSV-induced early signaling events in A549 AECs, while TLR3 pathway functions later during RSV infection (Liu et al. 2007). Their activation by viral dsRNA leads to the activation of NF-κB, promoting NF-κB-dependent gene transcription, and to the phosphorylation and nuclear translocation of IRF3 and IRF7 transcription factors, inducing the expression of type I IFNs, including IFN-β (Yu and Levine 2011; Newton and Dixit 2012). Secreted type I IFNs bind to their receptors at the cell surface, activating Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways. Phosphorylated STAT1, STAT2 and IRF9 form IFN-stimulated gene factor 3 (ISGF3) complex, which translocates into the nucleus and binds to IFN-stimulated response element (ISRE) present in IFN-regulated genes, promoting their expression (Samuel 2001; Au-Yeung et al. 2013). RSV-elicited TLR3 expression has been shown to depend on RIG-I-induced IFN-β secretion (Liu et al. 2007). Similarly, RIG-I, IFIT 1 and ISG15 expressions have been proved to be activated by type I IFN signaling (Loeb and Haas 1992; Elco and Sen 2007; Yount et al. 2007; Zhou et al. 2013), even though ISG15 expression can also be triggered by dsRNA signaling through IRF3 activation by an IFN-independent mechanism and by NF-κB (reviewed in Jeon et al. 2010). TNFAIP3 expression has been shown to be controlled by NF-κB and CCAAT-enhancer box binding protein β (C/EBPβ) transcription factors (Ma and Malynn 2012; Lai et al. 2013). The fact that internalized synthetic surfactant vesicles decreased RSV-elicited expression of ISG15, IFIT 1, TNFAIP3, TLR3 and RIG-I (figure 4) suggests that they must be inhibiting some step of the signaling pathways that activate IRF3-, NF-κB- or JAK/STAT-dependent transcription.

Concerning the activation of cellular pathways, internalized synthetic surfactant vesicles were demonstrated to inhibit RSV-induced activation of the PI3K/Akt pathway (figure 6) and ERK MAPK (figure 7). However, they showed no regulatory effect on the activation of NF-κB canonical pathway or p38 MAPK (figure 7B).

The two RSV nonstructural proteins NS1 and NS2 were proved to mediate RSV-induced activation of the PI3K/Akt pathway in A549 AECs, suppressing premature apoptosis of infected cells and therefore improving viral growth (Thomas et al. 2002; Bitko et al. 2007). Thus, the inhibition of the PI3K/Akt pathway by internalized synthetic surfactant vesicles could counteract this RSV-elicited anti-apoptotic mechanism. In

addition to that, internalized surfactant vesicles were already proved to down-regulate the activation of the PI3K/Akt pathway in LPS-stimulated murine AMs (chapter 2). Nonetheless, the mechanism by which they accomplish this effect is still unknown. Class I PI3Ks generate phosphatidylinositol-3,4,5-triphosphate and indirectly phosphatidylinositol-3,4-biphosphate, which bind to the pleckstrin homology domain of Akt, promoting its phosphorylation and activation by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) (Vanhaesebroeck et al. 2012). Our results do not allow us to determine which of these signaling steps is regulated by internalized synthetic surfactant vesicles.

Moreover, the inhibition of the PI3K/Akt pathway could mediate the down-regulation of RSV-elicited expression of inflammatory mediators. In a previous report, inhibition of this pathway in RSV-infected A549 AECs did not modify NF-κB nuclear translocation, but significantly decreased NF-κB-dependent gene transcription (Thomas et al. 2002). More experiments need to be performed to discover a possible relationship between the inhibition of the PI3K/Akt pathway and the decrease in the expression of inflammatory markers in RSV-infected A549 cells.

NF-κB is a central mediator of RSV-induced airway inflammation. RSV infection has been shown to induce NF-κB activation by three different pathways in A549 AECs. First of all, RSV activates NF-κB canonical pathway, in which NF-κB inhibitor protein IκB-α is phosphorylated and targeted for proteosomal degradation by IκB kinase (IKK) complex, composed of IKKα, IKKβ and IKKγ. As a result, RelA (p65)/p50 complexes are released to enter the nucleus and activate NF-κB-dependent gene transcription (Garofalo et al. 1996; Jamaluddin et al. 1998). Secondly, recognition of RSV dsRNA by RIG-I triggers the activation of NF-κB noncanonical pathway, in which a complex of NF-κB-inducing kinase (NIK) and IKKα activates posttranslational processing of the 100 kDa precursor of p52. Newly formed p52 binds to RelB and both translocate into the nucleus (Choudhary et al. 2005; Liu et al. 2008). Finally, the third pathway is a cross-talk pathway between the other two, in which RIG-I-induced activation of NIK/IKKα complex promotes the release of RelA from a p100/RelA complex and its nuclear translocation (Liu et al. 2008). Some authors have suggested that the noncanonical pathway functions at early stages of RSV infection, while the canonical pathway predominates later (Choudhary et al. 2005). In fact,

they did not detect IκB-α proteolysis until 24 hours after RSV adsorption (Jamaluddin et al. 1998). Consistent with this, we could not detect IκB-α phosphorylation in A549 cells infected for 2.5 hours with RSV (figure 7B). However, other groups have observed IκB-α phosphorylation 5 hours after RSV infection of A549 AECs (Bitko et al. 2007). In any case, the effect of internalized synthetic surfactant vesicles on the activation of NF-κB noncanonical and canonical pathways should be further analyzed at early and late time points after RSV-infection, respectively.

RSV infection has also been reported to elicit the activation of p38 and ERK MAPKs in A549 AECs. Even though the authors did not find an increase in p38 phosphorylation levels after RSV infection, they demonstrated that the virus was able to stimulate p38 catalytic activity at 3 hours post-infection (Pazdrak et al. 2002). Similarly, we could not detect an induction of p38 phosphorylation levels at 2.5 hours after RSV infection (figure 7B). p38 catalytic activity should be analyzed in future experiments in order to examine whether RSV-infection is able to induce p38 activation in our A549 cells, and whether internalized synthetic surfactant vesicles can regulate it.

On the other hand, we found that ERK phosphorylation levels were increased in RSV-infected A549 AECs and that internalized surfactant lipid component down-regulated this increase (figure 7). ERK activation in RSV-infected cells is mediated by different pathways (Monick et al. 2004; Johnson et al. 2012). For example, the activation of TLR3 and RIG-I by viral dsRNA or the activation of TLR4 by RSV F glycoprotein triggers the activation of ERK, p38 and JNK MAPKs (Kurt-Jones et al. 2000; Liu et al. 2007; Yu and Levine 2011; Newton and Dixit 2012). Additionally, RSV infection was also proved to activate different protein kinase C (PKC) isoforms, including PKCδ, which subsequently activated ERK MAPK in A549 AECs (Monick et al. 2001). Interestingly, one of synthetic surfactant lipids, DPPC, attenuated LPS-induced respiratory burst in a human monocytic cell line via downregulation of PKCδ isoform. This effect appeared to be dependent on DPPC uptake by the cells (Tonks et al. 2005). More experiments need to be conducted in order to elucidate the mechanism underlying the immunoregulatory action of internalized surfactant lipids on RSV-elicited ERK activation in A549 AECs.

Among synthetic surfactant components, surfactant lipids were demonstrated to be responsible for the down-regulation of ISG15 and RIG-I expressions and ERK activation in

RSV-infected cells (figures 5 and 7). However, a possible immunomodulatory action of internalized human recombinant SP-C in RSV-infected A549 cells cannot be ruled out. Studies performed with SP-C-deficient mice proved that this protein plays an essential role in the immune response against RSV (Glasser et al. 2009; Glasser et al. 2013a). For example, TLR3 expression was increased in the lungs of SP-C-deficient mice compared with control mice before and after RSV infection (Glasser et al. 2009). This suggests that SP-C could be involved in the inhibition of TLR3 expression. Furthermore, endocytosed SP-C was proved to modulate the expression of pro- and anti-inflammatory markers in MH-S murine AMs (chapter 2). This synthetic surfactant based on recombinant human SP-C represents a useful tool to study the possible immunoregulatory action exerted by SP-C during RSV infection.

Altogether, our results demonstrate for the first time that internalized vesicles of a synthetic surfactant based on recombinant human SP-C exert an immunoregulatory effect on RSV-infected A549 AECs. This means that the protective function of surfactant components is not restricted to the extracellular medium, and reinforces the use of surfactant-based therapies in RSV-infected high risk individuals. Furthermore, understanding the mechanism by which surfactant exerts its immunoregulatory effect could help the development of new antiviral drugs or the discovery of new pharmacological targets to counteract RSV-induced inflammation.

GENERAL DISCUSSION

The **main objective** of this thesis was to study the immunoregulatory properties of a synthetic surfactant based on recombinant human surfactant protein C (SP-C) and to unravel new details about the mechanism of action of each of its components. This synthetic surfactant (sSPC) is composed of dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylglycerol (POPG), palmitic acid (PA) and recombinant human SP-C. The results presented in this thesis provide new evidences about the immunomodulatory action exerted by sSPC. Furthermore, each of sSPC components was demonstrated to play a different and essential role in this immunoregulatory effect, depending on their extracellular or intracellular location.

Synthetic surfactants are currently being developed to treat neonatal respiratory distress syndrome (RDS), a pathology mainly caused by lung immaturity and surfactant deficiency (Whitsett and Weaver 2002). The synthetic surfactant based on recombinant human SP-C has been shown to be effective in animal models of lung injury (Lewis et al. 1999; Spragg et al. 2000; Ikegami and Jobe 2002) and treating acute respiratory distress syndrome (ARDS) patients (Spragg et al. 2003; Spragg et al. 2004; Markart et al. 2007). One of these studies reported a potential anti-inflammatory action of the synthetic surfactant based on recombinant human SP-C, since the treatment with sSPC decreased the levels of the pro-inflammatory cytokine interleukin (IL)-6 in bronchoalveolar lavages of patients suffering ARDS (Spragg et al. 2003). In addition to that, sSPC was also shown to reduce the expression of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) and to enhance the production of the immunosuppressive cytokine IL-10 in a human monocytic cell line stimulated with bacterial lipopolysaccharide (LPS) (Wemhöner et al. 2009). However, the role of each component and their mechanism of action had not been analyzed yet.

The **first chapter** of this thesis evaluated the extracellular anti-inflammatory effect of the synthetic surfactant based on recombinant human SP-C on the inflammatory response of LPS-stimulated mouse alveolar and peritoneal macrophages. To accomplish this task, we used two murine macrophage cell lines: MH-S alveolar macrophages (AMs) and RAW 264.7 peritoneal macrophages. The cells were stimulated with LPS in the presence or absence of synthetic surfactant membranes containing human recombinant SP-C (sSPC), or surfactant membranes without SP-C (sPL).

Synthetic surfactant vesicles were demonstrated to disrupt LPS-induced pro-inflammatory response in MH-S murine AMs, inhibiting the three key signaling pathways activated by LPS (mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K) and nuclear factor-κB (NF-κB) cascades) as well as LPS-elicited production of the pro-inflammatory mediators TNF-α and inducible nitric oxide synthase (iNOS). This immunomodulatory action was not restricted to MH-S AMs, since sPL and sSPC vesicles were also shown to attenuate TNF-α secretion in RAW 264.7 mouse peritoneal macrophages. This anti-inflammatory effect took place in the extracellular medium, because it disappeared as surfactant vesicles were endocytosed by the cells through a clathrin-dependent mechanism. The results obtained also indicated that synthetic surfactant vesicles did not trap LPS molecules, and that they carried out their immunoregulatory action directly on the surface of AMs, probably by interfering with LPS binding to its receptors on the surface of the cells.

Among synthetic surfactant components, SP-C attenuated only iNOS mRNA production and protein levels, indicating that surfactant lipids were responsible for the disruption of LPS-induced pro-inflammatory response. More precisely, we determined that vesicles without POPG were unable to inhibit TNF-α release after LPS stimulation. Therefore, the extracellular anti-inflammatory action of synthetic surfactant vesicles on LPS-induced response in MH-S AMs was exerted by POPG.

POPG immunoregulatory properties have already been described. Segregated pools of POPG have been shown to suppress respiratory syncytial virus (RSV) and influenza A virus infections (Numata et al. 2010; Numata et al. 2012a; Numata et al. 2013a; Numata et al. 2013b), to disrupt Toll-like receptor (TLR)2 ligand-induced pro-inflammatory responses (Kandasamy et al. 2011) and to inhibit TNF-α and nitric oxide productions as well as activation of MAPK and NF-κB pathways elicited by LPS in primary rat and human AMs and in U937 cells, by binding to CD14 and MD2 proteins (Kuronuma et al. 2009). In a previous report, POPG was also proved to inhibit the binding of LPS to LPS-binding protein (LBP) and CD14 (Hashimoto et al. 2003). Accordingly to our results, in all these studies, the presence of POPG in the extracellular medium was essential for its anti-inflammatory action. Additionally, in our work, we have demonstrated for the first time that POPG is also able to regulate LPS-induced activation of the PI3K/Akt pathway. Synthetic

surfactant POPG interaction with CD14, MD2 or LBP would be consistent with all our previous results, as it would promote the inhibition of all LPS-activated signaling pathways through an extracellular mechanism.

Moreover, observation of rhodamine-phosphatidylethanolamine-labeled DPPC/POPG and DPPC/POPG/PA giant unilamellar vesicles (GUVs) by fluorescence microscopy revealed that the presence of PA in DPPC/POPG/PA (sPL) vesicles enhanced their anti-inflammatory action by increasing ordered/disordered phase segregation and enrichment of POPG in disordered domains. Actually, PA is a common additive in replacement surfactants because it increases their surface activity (Sáenz et al. 2006; Mingarro et al. 2008). Our results proved for the first time that the presence of PA or other segregating compounds is essential for increasing ordered/disordered phase segregation in surfactant membranes and improving synthetic surfactant POPG immunoregulatory properties.

In this study, DPPC did not exert any modulatory action on LPS-induced response in AMs. However, DPPC has been demonstrated to inhibit LPS-elicited IL-8 production in A549 lung epithelial cells by blocking TLR4 translocation into lipid raft domains (Abate et al. 2010). Furthermore, it has been proved to decrease the biosynthesis of platelet-activating factor after LPS stimulation (Tonks et al. 2003), and to attenuate LPS-induced respiratory burst via down-regulation of protein kinase C (PKC) δ isoform (Tonks et al. 2005) in a human monocytic cell line. Interestingly, in these studies the maximum effect was observed after preincubating DPPC with the cells for 2 hours, and DPPC immunoregulatory properties appeared to be dependent on its uptake by the cells. Taking this information into account, we hypothesized that synthetic surfactant components like POPG or DPPC could have different effects depending on their cellular location.

In fact, in the alveolar space surfactant is endocytosed by AMs and type II pneumocytes in order to assure its degradation and recycling (Casals and Cañadas 2012; Agassandian and Mallampalli 2013). The influence of these internalized surfactant components on the phenotype or the immune response of AMs and alveolar epithelial cells (AECs) remained largely unknown.

Therefore, the purpose of **the second chapter** of this thesis was to analyze the intracellular immunomodulatory action of synthetic surfactant vesicles internalized by AMs on the activation state of these cells after being stimulated with LPS. MH-S murine AMs

were preincubated with sSPC or sPL vesicles for 18 hours before being stimulated with LPS. This preincubation time was long enough to allow surfactant endocytosis, as proved by confocal microscopy studies.

Our results indicated that both human recombinant SP-C and the lipid component present in synthetic surfactant membranes exerted different intracellular immunomodulatory actions once internalized by MH-S cells, balancing the expression of pro- and anti-inflammatory mediators and limiting LPS-induced pro-inflammatory response.

On one hand, internalized synthetic surfactant lipids decreased LPS-elicited TNF-α (pro-inflammatory cytokine) expression and secretion and IL-10 (anti-inflammatory cytokine) release, being this effect very pronounced in the case of TNF-α release (78 ± 3% inhibition). In contrast, endocytosed sPL vesicles also increased LPS-induced expression of four pro-inflammatory markers (interferon regulatory factor (IRF)1, C-X-C motif chemokines 10 and 11 (CXCL10 and CXCL11) and CD80) and of two mediators that can have pro- or anti-inflammatory activity depending on their environment (cyclooxygenase 2 (COX2) and suppressor of cytokine signaling 3 (SOCS 3)).

The analysis of possible signaling pathways involved in these results proved that internalized sPL vesicles inhibited LPS-induced activation of NF-κB, as well as basal and LPS-elicited Akt phosphorylation and activation. Moreover, they promoted glycogen synthase kinase 3 (GSK3) activation in the absence or presence of LPS, probably as a consequence of the inhibition of Akt. However, they did not modify the activation of extracellular signal-regulated kinase (ERK) and p38 MAPKs in LPS-stimulated cells. The results obtained in experiments in which GSK3 was inhibited by lithium chloride demonstrated for the first time that the immunoregulatory action of internalized surfactant lipids is mediated at least in part by GSK3 activation, probably as a consequence of the inhibition of PI3K/Akt pathway.

Although our results do not allow us to distinguish which of synthetic surfactant lipids is responsible for these intracellular immunomodulatory actions, they agree with previous studies showing that DPPC attenuated LPS-induced respiratory burst in a human monocytic cell line without affecting ERK or p38 MAPK signaling after being taken up by the cells (Tonks et al. 2001). Instead, this effect was proved to be mediated by the down-regulation

of PKCδ isoform (Tonks et al. 2005). The fact that internalized synthetic surfactant lipids promoted the activation of GSK3 represents a link between our work and the results obtained by Tonks et al., because GSK3 has been shown to inhibit PKCδ activity (Wang et al. 2011). Taking these published observations into consideration, the possible role of DPPC in the intracellular immunoregulatory effect of sPL vesicles should be further analyzed. However, even though the anti-inflammatory action of POPG has only been proved when the lipid is in the extracellular medium, its possible role in the immunomodulatory action of internalized synthetic surfactant vesicles cannot be ruled out yet.

On the other hand, human recombinant SP-C endocytosed by MH-S murine AMs specifically up-regulated the expression of two molecules involved in the development of alternative immune responses (IL-4 and CD200 receptor 3 (CD200R3)) as well as LPS-elicited production of the pro-inflammatory marker iNOS. This represents a difference with the results obtained in the first chapter, which proved that SP-C present in extracellular surfactant membranes attenuated LPS-induced iNOS production. More experiments need to be conducted in order to explain these opposite results and to determine which signaling pathways are being regulated by SP-C in the extracellular and in the intracellular medium.

In the alveolar space, surfactant is also endocytosed by type II AECs (Agassandian and Mallampalli 2013). These cells have been recognized to be involved in the immune defense of the lungs against various pathogens, including RSV (Zhang et al. 2001; Unkel et al. 2012; Chuquimia et al. 2013). RSV is a common and highly contagious respiratory virus that infects airway epithelial cells and other structural and resident immune cells, inducing a strong pro-inflammatory response which promotes virus clearance but can also be detrimental to the host (Lotz and Peebles 2012). The effect of synthetic surfactant components once endocytosed by type II AECs on their immune response against RSV had not been studied yet.

Therefore, the aim of the **third chapter** of this thesis was to examine the intracellular immunoregulatory functions of internalized synthetic surfactant vesicles on the immune response of A549 AECs infected with human RSV. A549 cells are human epithelial cells that retain morphological features of type II AECs (Lieber et al. 1976). They were

preincubated with sPL and sSPC vesicles for 18 hours before being mock-infected or infected with human RSV.

Analysis of SP-C protein levels in A549 cells previously preincubated with sSPC vesicles for 18 hours and subsequently mock-infected or infected with RSV for 24 hours revealed that SP-C had been taken up by these cells. Thus, we concluded that 18 hours was a time long enough to allow the internalization of synthetic surfactant vesicles by A549 AECs.

Our results provided evidence that internalized synthetic surfactant vesicles exerted an intracellular anti-inflammatory action on RSV-infected A549 AECs. Even though they were unable to inhibit viral replication, they decreased RSV-induced expression of two antiviral molecules (interferon-stimulated gene 15 (ISG15) and interferon-induced protein with tetratricopeptide repeats (IFIT) 1), of a protein that inhibits NF-κB and IRF3 transcription factors (tumor necrosis factor α-induced protein 3 (TNFAIP3)), and of two cellular receptors that recognize viral double stranded RNA (TLR3 and retinoic acid-inducible gene I (RIG-I)). They also attenuated RSV-elicited activation of the PI3K/Akt pathway and ERK MAPK. However, they showed no modulatory effect on the activation of the NF-κB canonical pathway or p38 MAPK. Moreover, surfactant lipids were found to be responsible for the down-regulation of ISG15 and RIG-I expressions and ERK activation in RSV-infected cells.

The results presented in chapters 2 and 3 demonstrated for the first time that internalized synthetic surfactant vesicles can inhibit the activation of the PI3K/Akt pathway in both LPS-stimulated MH-S AMs and in RSV-infected A549 AECs. However, more experiments should be conducted in order to confirm if internalized surfactant lipids are responsible for this effect not only in LPS-stimulated MH-S AMs but also in A549 cells infected with RSV. In addition to that, internalized sPL vesicles attenuated NF-κB activation but not ERK phosphorylation in LPS-stimulated MH-S AMs, whereas in RSV-infected A549 AECs the opposite effect was observed. This difference reveals that the signaling pathways regulated by internalized synthetic surfactant lipids are cell- and pathogen-specific.

Even though the results obtained in the third chapter did not prove it, a possible immunomodulatory action of internalized human recombinant SP-C on RSV-infected A549

cells cannot be ruled out. Studies performed with SP-C-deficient mice showed that this protein plays an essential role in the immune response against RSV (Glasser et al. 2009; Glasser et al. 2013a). For example, TLR3 expression was increased in the lungs of SP-C-deficient mice compared with control mice before and after RSV infection (Glasser et al. 2009). This suggests that SP-C could be involved in the inhibition of TLR3 expression.

Altogether, the results presented in this thesis provide evidence of the different immunoregulatory actions exerted by synthetic surfactant components on LPS-stimulated AMs and on RSV-infected A549 AECs, which depend on their intra or extracellular location. However, the structure and size of synthetic surfactant vesicles were also demonstrated to influence their immunoregulatory properties. In the first chapter, synthetic surfactant multilamellar vesicles (MLVs) were shown to have less anti-inflammatory properties than small unilamellar vesicles (SUVs) on the response of AMs to LPS. This is probably due to the fact that POPG molecules in the inner bilayers of MLVs are not exposed to the extracellular medium. Moreover, surfactant MLVs were not internalized by MH-S AMs to the same extent than SUVs (chapter 2), which were proved to be endocytosed by MH-S cells through a clathrin-dependent mechanism (chapter 1). This is physiologically relevant since exogenous surfactants are currently delivered to patients in the form of MLVs in order to mimic the structure of large surfactant aggregates, which have high surface activity and adsorb very rapidly to the air-liquid interface. Nevertheless, surface compression and expansion cycles generate small vesicles that have poor surface activity and are endocytosed by AMs and type II AECs (Casals and Cañadas 2012). Therefore, administration of this synthetic surfactant as MLVs would improve lung function preserving its extracellular and intracellular immunoregulatory properties.

Surfactant replacement therapy is currently the standard of care for the prevention and treatment of neonatal RDS. It is also routinely used to treat newborns with meconium aspiration syndrome as well as infants with respiratory failure from pneumonia. However, the efficacy and clinical benefits of surfactant therapy in acute lung injury (ALI)/ARDS or in RSV-bronchiolitis are not completely demonstrated (Barreira et al. 2011; Willson and Notter 2011). Moreover, the use of exogenous surfactants as anti-inflammatory or immunomodulatory agents during lung inflammatory diseases has not been approved yet. Altogether, the results presented in this thesis reveal new details about the

immunoregulatory actions exerted by the synthetic surfactant based on recombinant human SP-C on LPS-stimulated AMs and on RSV-infected A549 AECs. Furthermore, they highlight the different roles played by each of sSPC components in this immunoregulatory effect. Understanding the mechanism by which surfactant components are able to regulate lung-immune responses could help to improve synthetic surfactant formulations and to develop new anti-inflammatory drugs to counteract pulmonary inflammatory diseases.

CONCLUSIONS

The research presented in this doctoral thesis provides new evidences about the immunoregulatory properties of the synthetic surfactant based on recombinant human surfactant protein C (SP-C) and reveals further details about the mechanism of action of each of its components. Taking all the results into consideration, we conclude that:

- Synthetic surfactant lipid component, and specifically palmitoyloleoylphosphatidylglycerol (POPG), is responsible for the disruption of the pro-inflammatory response of lipopolysaccharide (LPS)-stimulated alveolar macrophages (AMs). This effect is carried out in the extracellular medium, since it disappears as the vesicles are endocytosed by the cells. Considering that synthetic surfactant vesicles do not trap LPS molecules, their anti-inflammatory action seems to take place directly on the surface of AMs, probably by interfering with LPS binding to its receptor; which explains the blockage exerted by the synthetic surfactant on all LPS-activated signaling pathways. On the other hand, recombinant human SP-C attenuates only LPS-elicited inducible nitric oxide synthase (iNOS) production. Our results also demonstrated for the first time that the presence of palmitic acid (PA) is essential for increasing ordered/disordered phase segregation in synthetic surfactant membranes, enriching POPG in disordered domains and thereby improving its immunoregulatory properties.

- Once endocytosed by AMs, both human recombinant SP-C and the lipid component present in synthetic surfactant membranes exert intracellular immunomodulatory actions, balancing the expression of pro- and anti-inflammatory mediators that modulate the response of the cells to LPS. The final result is the limitation of the pro-inflammatory response induced by the endotoxin. Our results also demonstrated for the first time that the immunoregulatory effect of internalized surfactant lipids is mediated at least in part by glycogen synthase kinase 3 (GSK3) activation.

- Internalized synthetic surfactant vesicles exert also an intracellular anti-inflammatory action on respiratory syncytial virus (RSV)-infected A549 alveolar epithelial cells, inhibiting RSV-induced expression of inflammatory mediators, as

well as RSV-elicited activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK). Moreover, internalized synthetic surfactant lipid component has been demonstrated to be responsible for the down-regulation of interferon-stimulated gene 15 (ISG15) and retinoic acid-inducible gene I (RIG-I) expressions and ERK activation in RSV-infected cells.

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